Immune Dysfunction Following Severe Polytrauma & Major Surgery: Exploring Mechanisms & Identifying Potential Therapies

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Statement of originality

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Published Works, Prizes & Awards

Published Works From This Thesis

1. 'Does major surgery induce immune suppression and increase the risk of postoperative infection?' **Torrance HD**, Pearse RM, O'Dwyer MJ. *Current Opinion in Anesthesiology*. (*ePub 9 Mar)* 2016. PMID*:* 26963469

2. 'Epigenetic regulatory pathways involving micro-RNAs may modulate the host immune response following major trauma' Owen HC, **Torrance HD**, Jones TF, Pearse RM, Hinds CJ, Brohi K, O'Dwyer MJ. *Journal of Trauma and Acute Care Surgery*. 2015; 79:766-72. PMID: 26496100

3. 'Features of postoperative immune suppression are reversible with interferon gamma and independent of IL-6 pathways' Longbottom ER*, **Torrance HD***, Owen HC, Fragkou PC, Pearse RM, Hinds CJ, O'Dwyer MJ. *Annals of Surgery.* (ePub Oct 2015) PMID: 26445474 (***Joint First Authors**)

4. 'The perioperative immune response' O'Dwyer MJ, Owen HC, **Torrance HD**. *Current Opinion in Critical Care*. 2015; 21:336-42. PMID: 26103142

5. 'Systemic Inflammatory Response Syndrome After Major Abdominal Surgery Predicted by Early Upregulation of TLR4 and TLR5' Lahiri R, Derwa Y, Bashir Z, Giles E, **Torrance HD**, Owen HC, O'Dwyer MJ, O'Brien A, Stagg AJ, Bhattacharya S, Foster GR, Alazawi W. *Annals of Surgery.* (ePub May 2015) PMID: 26020106

6. 'Changes in gene expression following trauma are related to the age of transfused packed red blood cells' **Torrance HD**, Vivian ME, Brohi K, Prowle JR, Pearse RM, Owen HC, Hinds CJ, O'Dwyer MJ. *Journal of Trauma and Acute Care Surgery*. 2015; 78:535-42. PMID: 25710424

7. ‘Perioperative blood transfusion is associated with a gene transcription profile characteristic of immunosuppression: a prospective cohort study’ Fragkou PC*, **Torrance HD***, Pearse RM, Ackland GL, Prowle JR, Owen HC, Hinds CJ and O'Dwyer MJ. *Critical Care*. 2014; 18:541 PMID: 25270110 (***Joint First Authors**)

8. ‘Association between gene expression biomarkers of immunosuppression and blood transfusion in severely injured polytrauma patients’ **Torrance HD**, Brohi K, Pearse RM, Mein CA, Wozniak E, Prowle JR, Hinds CJ, O’Dwyer MJ. *Annals of Surgery*. 2015; 261:751-9. (ePub Mar 2014) PMID: 24670848

Prizes & Awards

1. Rosetrees Trust, Research Prize, 2015.

2. Royal College of Surgeons of England, Galeski & Lawrie Fellowship, 2014.

3. Intensive Care Society, Best Research Poster Presentation Award, 2013.

4. London Trauma Conference, Best Oral Presentation, 2013.

5. Royal College of Surgeons of England, One-Year Research Fellowship, 2013.

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For my wife & my parents.

Abstract

Introduction

Following polytrauma and major surgery patients experience a period of immunosuppression, which can predispose them to the development of nosocomial infections. This thesis examines how polytrauma and surgery influences T-helper (T_h) cell differentiation and antigen presentation, whilst exploring how the detrimental immunomodulatory effects of these insults may be reversed.

Methods

Serial blood samples were drawn from two cohorts of patients at The Royal London Hospital; following severe polytrauma (*n=112*) or major abdominal surgery (*n=119*). mRNA levels of candidate cytokines and transcription factors were assayed, along with protein levels of key cytokines IL-10 and IL-6. Associations between these data, acquisition of nosocomial infection and outcome were described. As a validated surrogate of immune competence $CD14^{+}$HLA-DR levels were quantified. *In vitro* models explored the reversibility of tissue damage induced immunosuppression and determined the role of individual circulating mediators in altering host immune function.

Results

A consistent up-regulation in gene expression of prototypical anti-inflammatory pathways in conjunction with features of depressed pro-inflammatory T_h cell pathways was detected across both cohorts. This was accompanied by early down-regulation of $CD14^{+}$HLA-DR. Gene expression changes were quantitatively associated with the subsequent acquisition of nosocomial infections. Allogeneic blood transfusion exacerbated these findings

and was independently associated with an increased risk of nosocomial infection. Culture experiments determined that postoperative decreases in antigen presentation were IL-10 dependent and reversible in the presence of Interferon-Gamma and Granulocyte Monocyte-Colony Stimulating Factor.

Conclusions

This thesis describes a significant host immune response immediately following significant tissue damage which is dominated by features of immune suppression. Blood transfusion appears to have a distinct, additive effect. These data identify a potential role for targeted treatment with currently licenced immune stimulants (IFN-γ and GM-CSF). In addition exploitation of the IL-10 signalling pathway may be of importance as a strategy to reduce the incidence of nosocomial infections.

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Glossary Of Terms

Ab / C ..... Antibodies per Cell

ACCU ..... Adult Critical Care Unit

ACIT ..... Activation of Coagulation and Inflammation in Trauma

ACLS ..... Advanced Cardiac Life Support

AIS ..... Abbreviated Injury Score

APTT ..... Activated Partial Thromboplastin Time

ARDS ..... Acute Adult Respiratory Distress Syndrome

ASA ..... American Society of Anaesthesiologists

ATC ..... Acute Traumatic Coagulopathy

ATLS ..... Advanced Trauma Life Support

ATP5B ..... ATP synthase subunit beta

B2M ..... β2 microglobulin

BD ..... Base Deficit

BSI ..... Blood Stream Infection

CARS ..... Compensatory Anti-Inflammatory Response Syndrome

CD ..... Cluster Differentiation

CDC ..... Center for Disease Control

cDNA ..... Complementary Deoxyribonucleic Acid

CLRN ..... Comprehensive Local Research Network

CPET ..... Cardio Pulmonary Exercise Test

C_t ..... Threshold Cycle

CTLA-4 ..... Cytotoxic T-lymphocyte-Associated Protein-4

DAMP ..... Damage Activated Molecular Pattern

DO_2 ..... Oxygen Delivery

ED ..... Emergency Department

EDTA..... Ethylene-Diaminete-Traacetic Acid

ELISA ..... Enzyme Linked Immunosorbant Assay

ERK ..... Extracellular Signal-Regulated Kinases

ESBL ..... Extended-Spectrum Beta-Lactamases

FACS ..... Fluorescence-activated cell sorting

FBC..... Fetal Bovine Serum

FFP..... Fresh Frozen Plasma

FOXP3 ..... Forkhead Box P3

FRET ..... Fluorescence Resonance Energy Transfer

GAPDH ..... Glyceraldehyde 3-phosphate dehydrogenase

GATA-3..... GATA Binding Protein 3

GCP ..... Good Clinical Practice

GM-CSF ..... Granulocyte Macrophage Colony-Stimulating Factor

gMFI ..... geometric Mean Fluorescent Intensity

HEMS ..... Helicopter Emergency Medical Service

HLA-DR ..... Human Leukocyte Antigen DR

HMGB1 ..... High-Mobility-Group Box 1

HPB ..... Hepato-Pancreato-Biliary

HR(s) ..... Hour(s)

HTS..... Hypertonic Saline

ICNARC ..... Intensive Care National Audit and Research Centre

ICU ..... Intensive Care Unit

IFN-γ..... Interferon Gamma

IgG ..... Immunoglobulin G

IL ..... Interleukin

ILC..... Innate Lymphoid Cells

IQR ..... Interquartile Range

ISS ..... Injury Severity Score

LAR ..... Legally Appointed Representative

LAS..... London Ambulance Service

M..... Average Expression Stability

Mac-1..... Macrophage-1 Antigen

MAPK..... Mitogen Activated Protein Kinase

MCT..... Micro-Centrifuge Tube

MDSC..... Myeloid Derived Suppressor Cell

MET..... Metabolic Equivalent

MHC ..... Major Histocompatibility Complex

miRNA..... Micro Ribonucleic Acid

MOD..... Multi Organ Dysfunction

mRNA..... Messenger Ribonucleic Acid

MRSA..... Methicillin-Resistant Staphylococcus aureus

MSSA ..... Methicillin-Sensitive Staphylococcus aureus

mtDNA ..... Mitochondrial Deoxyribonucleic Acid

MVC ..... Motor Vehicle Collision

MYD88..... myeloid differentiation primary-response protein 88

NCEPOD ..... National Confidential Enquiry into Patient Outcome and Death

NF-κB ..... Nuclear Factor Kappa-light-chain-enhancer of activated B cells

NFW ..... Nucleotide Free Water

NHS ..... National Health Service

NK ..... Natural Killer

NLR ..... NOD-Like Receptor

nm ..... Nano Metre

NSAID..... Non-Steroidal Anti-Inflammatory Drug

OR..... Odds Ratio

PAMP ..... Pathogen Activated Molecular Pattern

PBMC ..... Peripheral Blood Mononuclear Cell

PBS ..... Phosphate Buffered Saline

PD-1..... Programmed Cell Death Protein-1

PE..... Phosphatidylethanolamine

PK ..... Proteinase K

PLT ..... Platelets

PRBC ..... Peripheral Blood Mononuclear Cell

PRR..... Pattern Recognition Receptors

PT..... Processing Tube

qRT-PCR ..... Quantitative Real-Time Polymerase Chain Reaction

RCF..... Relative Centrifugal Force

REBOA..... Resuscitative Balloon Occlusion of the Aorta

RIN ..... RNA Integrity Number

RNFW..... RNA-Free Water

RORγT..... RAR-related Orphan Receptor Gamma T

ROS ..... Reactive Oxygen Species

ROTEM ..... Rotational Thromboelastometry

RPMI ..... Roswell Park Memorial Institute

SAGM..... Sodium Chloride, Adenine, Glucose, Mannitol

SBP ..... Systolic Blood Pressure

SIRS..... Systemic Inflammatory Response Syndrome

SOCS ..... Suppressor of Cytokine Signalling

SOFA ..... Sequential Organ Failure Assessment

SSI ..... Surgical Site Infection

SST ..... Serum Separator Tube

STAT ..... Signal Transducer And Activator Of Transcription

TARN ..... Trauma Audit & Research Network

T-bet..... Transforming Growth Factor Beta

TBI..... Traumatic Brain Injury

TGF-β ..... Transforming Growth Factor-Beta

T_h ..... T-Helper Cell

TIC..... Trauma Induced Coagulopathy

TLR..... Toll-Like Receptors

TNF-α ..... Tumour necrosis factor-Alpha

T_{reg} ..... Regulatory T-Cell

TRIF..... TIR-domain-containing adaptor protein inducing IFN-β

TRIM ..... Transfusion Related Immune Modulatory

TT ..... Thrombin Time

UBC ..... Ubiquitin C

UK ..... United Kingdom

UKCRN ..... United Kingdom Clinical Research Network

UTI..... Urinary Tract Infection

VISION..... Vascular Events in Non-Cardiac Surgery

VRE ..... Vancomycin-Resistant Enterococcus

Chapter One: Introduction

The Chapter contains two published works:

'Does major surgery induce immune suppression and increase the risk of postoperative infection?' **Torrance HD**, Pearse RM, O'Dwyer MJ. *Current Opinion in Anaesthesiology*. (*ePub 9 Mar)* 2016. PMID: 26963469

'The perioperative immune response' O'Dwyer MJ, Owen HC, **Torrance HD**. *Current Opinion in Critical Care*. 2015; 21:336-42. PMID: 26103142

As a consequence in this Chapter aspects outlining the inflammatory response to tissue damage and subsequent risk of nosocomial infections have been co-authored by; Dr Hew DT Torrance, Dr Michael J O'Dwyer, Dr Helen C Owen & Professor Rupert M Pearse.

1.1 Trauma: A Disease Of Physical Injury

Trauma is the disease of physical injury (Trunkey *et al.*, 1974). Despite heightened public awareness of the catastrophic implications of severe trauma following recent conflicts in Iraq and Afghanistan it is primarily a civilian disease. Trauma itself can take the form of a diverse array of incidents such as; falls from height, motor vehicle collisions (MVC), stabbings or gunshot wounds. The disease affects both the western and developing worlds, however there is often a disparity, with a predominantly blunt mechanism of injury seen most often in the United Kingdom (UK), (National Audit Office, 2010). It is often cited in the literature as the most common disease affecting those under the age of 45 worldwide (Polinder *et al.*, 2012), however this paradigm is evolving as there is now a growing incidence of trauma affecting those over 60 (McCullough *et al.*, 2014). In the UK there are thought to be 20,000 episodes of major trauma per year which are believed to be responsible for 5,400 deaths (National Audit Office, 2010). However, as many more people suffer from serious lasting injury from their trauma this mortality figure doesn't address the impact that this disease has on the emotional, physical or psychological needs of these patients (Brohi *et al.*, 2011; Polinder *et al.*, 2012) or the complex rehabilitation and health requirements often needed by elderly trauma patients (McCullough *et al.*, 2014).

The estimated annual lost economic output due to deaths and serious injury from major trauma is thought to be between £3.3 and 3.7 billion (National Audit Office, 2010). This figure is however likely to be a gross underestimate, as the widespread, long lasting implications of trauma have already been alluded to. This figure predominantly reflects the injury and its immediate treatment, however significant amounts of NHS money are spent on the rehabilitation of these patients. From what little data we have on the rehabilitation phase, those suffering from trauma are often of lower socioeconomic groups (Roberts, 2012) and often have poor re-employment prospects (Brohi *et al.*, 2011; Soberg *et al.*, 2007). This is

clearly a topic that requires further research so we are able better to understand the impact of this disease on the UK population.

1.2 Changes To Trauma Management

1.2.1 The Birth Of ATLS

Over the last 30 years there have been progressive improvements in the provision of trauma care since the original opening of dedicated trauma centres in the United States in the 1960s. Often one of the most cited was the introduction of the Advanced Trauma Life Support (ATLS) programme. An American orthopaedic surgeon, who along with his family was tragically involved in a plane crash in Nebraska, established this course. When taking his severely injured family members to a local hospital he was appalled to find that he was able to provide better care for his family in the pre-hospital environment. As a result the ATLS programme was born, based on the successful Advanced Cardiac Life Support (ACLS) programme (Carmont, 2005). This basis for the assessment and management of trauma patients has been adopted worldwide with the ATLS manual now available in its 9th edition (American College of Surgeons, 2012). Interestingly, despite the impression that ATLS provides the gold standard of trauma care, there is no robust evidence demonstrating that its implementation has reduced trauma-related morbidity and mortality in the developed world (Jayaraman *et al.*, 2014; Wiles, 2015). With the rapid ongoing improvements to clinical practice the course or manual is not considered the cutting edge in trauma care; however it does provide a minimum competence, allowing a standardisation of the level of care for trauma patients to be applied worldwide (Wiles, 2015).

1.2.2 Recognition Of Acute Traumatic Coagulopathy

Alongside the structured management system that the ATLS programme provides there have been numerous developments in the resuscitation and operative strategies employed. A number of these have been developed by the United Kingdom and United States military

following their recent experiences in the Middle East (Hettiaratchy *et al.*, 2010). However one of the most important is the recognition and early treatment of coagulopathy. The first large-scale description of Acute Traumatic Coagulopathy (ATC) can be credited to Professor Karim Brohi from the Royal London Hospital (RLH) who published a landmark paper in the *Journal of Trauma* (Brohi *et al.*, 2003). This paper identified that nearly a quarter of polytrauma patients presenting to the Emergency Department (ED) were coagulopathic, (defined as a prothrombin time (PT) >18 seconds, activated partial thromboplastin time (APTT) >60 seconds, or thrombin time (TT) >15 seconds (1.5 times normal)), (Brohi *et al.*, 2003). Recognition of this life threatening pathology with novel point of care testing devices such as Rotational Thromboelastometry (ROTEM) has led to changes in the resuscitation strategy of these patients (Davenport *et al.*, 2011). This has led to a paradigm shift: Based largely on retrospective observational data there has been a movement away from the infusion of large volumes of clear fluids (as described in the previous ATLS guidelines (American College of Surgeons, 2012)) with an aim to replace any lost volume with a set ratio of blood, to blood products (Holcomb *et al.*, 2015). This has led to the inclusion of massive transfusion protocols in all western major trauma centres (MTCs) (Khan *et al.*, 2013; Nunez *et al.*, 2010). In addition to this there has been widespread adoption in Europe of the anti-fibrinolytic drug tranexamic acid, with early administration (<3 hours following injury) showing reductions in mortality in trauma patients (Crash-trial collaborators *et al.*, 2010).

1.2.3 Damage Control Resuscitation & Surgery

Damage Control Resuscitation is again a relatively recent phenomenon. Haemorrhage in trauma accounts for over a third of (potentially preventable) deaths (Gruen *et al.*, 2006; Sauaia *et al.*, 1995) and damage control resuscitation is based on two strategies, permissive hypotension and haemostatic resuscitation. 'Permissive' hypotension is based on the premise that aggressive infusion of fluids leads to the dilution of clotting factors, an increase in metabolic acidosis (due to the pH and chloride content of these clear fluids) and with the infusion of un-warmed fluids; hypothermia (Lamb *et al.*, 2014). This therefore may

exacerbate, what is termed the lethal triad of coagulopathy, acidosis and hypothermia (Holcomb *et al.*, 2007). Additionally, artificially raising the blood pressure to normotensive pressures can lead to disruption of the first fragile clot formed, causing bleeding to recur (Mitra *et al.*, 2012). This 'permissive' hypotension is a temporary measure, which is continued until there is control of the bleeding source. This obviously has to be carefully balanced with acceptable levels of oxygen delivery (DO_2) so as not to exacerbate any anaerobic respiration that may be present and cause cell necrosis and irreversible organ damage. This is paired with haemostatic resuscitation, the principle being that patients resuscitated with ratios closer to whole blood i.e. 1:1 or 1:2 of PRBCs to Fresh Frozen Plasma (FFP) alongside good quantities of platelets and cryoprecipitate were less likely to experience coagulopathy of mass transfusion (Lamb *et al.*, 2014). However there is controversy with these data as it is largely based on observational or retrospective data, with some critics citing a survival bias (Ho *et al.*, 2012). Further recent observational data also questions the effectiveness of current haemostatic resuscitation, as despite its use during the acute bleeding phase in trauma resuscitation and damage control surgery there were no improvements to patient hypoperfusion or coagulopathy (Khan *et al.*, 2014).

Damage control surgery is a modern turn of phrase for a strategy that has been practised for millennia (Schreiber, 2012). This combines ongoing resuscitation with essential surgery, coordinated by both the surgical and anaesthetic teams. Surgical procedures are limited to the minimum necessary, designed to prevent physiological exhaustion commonly associated with immediate definitive surgical care (Rotondo & Zonies, 1997). Patients are then transferred to the Critical Care department for optimisation of coagulation, temperature and pH, returning sometimes days later to the operating theatre for definitive surgical procedures such as pelvic fixation (Lamb *et al.*, 2014).

1.2.4 The Fusion Of Military & Civilian Practice

Recent military conflicts have driven improvements in the care of severe polytrauma patients, putting into practice these principles, with encouraging evidence (Hettiaratchy *et al.*, 2010). They have also lead to a re-introduction of tourniquets and haemostatic dressings in both military and civilian practice (Kelly *et al.*, 2008) as well as the re-introduction of devices such as the REBOA (Resuscitative Endovascular Balloon Occlusion of the Aorta) to temporarily stabilise the patient suffering from a complex pelvic vascular injury or torso injury, until definitive vascular repair or artery embolisation is available. This technique has the potential be used as a less invasive option to 'clam shell' thoracotomy (with distal aorta clamping) for uncontrollable haemorrhage, however it requires a greater evidence basis for widespread usage (Morrison *et al.*, 2016).

1.2.5 Improvements In Civilian Practice

In the UK there have been dramatic improvements in the delivery of trauma care following a damning report carried out by the National Confidential Enquiry into Patient Outcome and Death (NCEPOD), (NCEPOD, 2007). This has led to the centralisation of care for trauma patients with the creation of MTCs as well as improvements to the training of ambulance staff (McCullough *et al.*, 2014). This was pioneered in London with all patients suffering major trauma being screened in the pre-hospital environment and consequently being transported directly to one of the four new MTCs with peripheral hospitals being bypassed. A recent appraisal of this organisational change demonstrated significant improvements in the overall quality of care and survival for patients treated at these MTCs (Cole *et al.*, 2015).

1.2.6 Survival To The Critical Care Unit

Trauma deaths generally follow a tri-modal distribution, with patients dying either at the scene, in the first 24-48 hours (due to massive haemorrhage or from unsurvivable injuries) or later due to multi-organ failure and sepsis (Lord *et al.*, 2014). As has already been alluded

to, the implementation of modern trauma practices and a better understanding of the pathophysiology of severe trauma are resulting in the admission of a cohort of severely injured patients, who previously would not have survived to hospital admission. These advances in trauma care have led to a change in the perception of what an unsurvivable injury is. During the first 24-48 hours there have been improvements in the management of massive haemorrhage trauma induced coagulopathy. After arriving on the Critical Care unit the most serious threat to their survival is the development of nosocomial infections, sepsis and multi-organ failure (Lord *et al.*, 2014).

1.3 Major Abdominal Surgery: A Global Health Problem

Annually there are an estimated 234.2 million major surgical procedures performed globally (Weiser *et al.*, 2008). These procedures are performed in diverse health care settings, where there may be different standards of care (Pearse *et al.*, 2012). As a result there is a wide range in the incidence of complications reported in the literature. These complications are many; including death, infection, postoperative bleeding, deep vein thrombosis, stroke, cardiac injury and renal injury amongst others (Bratzler & Hunt, 2006). Due to the sheer number of patients undergoing operations worldwide even marginal gains to reduce morbidity and mortality could have dramatic population benefits.

1.3.1 Challenges For Major Elective Surgery

Therefore a challenge for clinicians is to minimise the mortality risk, as well as the morbidity of these perioperative complications. This is primarily done via the identification of the high-risk patient. It has been reported that the mortality rate for major elective surgery in the UK is as high as 4% for non-cardiac surgery (Pearse *et al.*, 2012). However this statistic doesn't tell the complete story, as 80% of these deaths occur in a much smaller cohort of only 12% of the surgical population (Pearse *et al.*, 2006). As a result research has been targeted towards identifying this at risk cohort by preoperative screening. This preoperative screening can take the form of non-invasive testing based on past medical

history and available blood tests, inputting these values into algorithms designed to define risk. This therefore allows clinicians to allocate appropriate levels of perioperative care, individualising treatment. The alternative option is via sophisticated tools designed to assess the individual patient's functional capacity, commonly cardiopulmonary exercise testing (CPET). This testing is based upon the premise that patients who were able to perform at four – six metabolic equivalents (METs) on testing had a reduced perioperative risk (Smith *et al.*, 2009). A large international study is ongoing to evaluate definitively the benefit of preoperative CPET in elective major surgery (UKCRN, 2013). However, all of these preoperative scoring systems are designed mainly to address mortality risk as well as key comorbidities such as cardiovascular disease or renal dysfunction and as a result poorly predict the incidence of nosocomial infection following elective major abdominal surgery.

1.3.2 Improvements To Surgical Care

Over the last twenty years there have been steady improvements to the care of elective surgical patients. These have led to the more judicious administration of fluids and vasoactive drugs in the perioperative period (Pearse *et al.*, 2011), with a recent meta-analysis suggesting that postoperative goal-directed therapy, the use of cardiac output monitors to guide the administration of intravenous fluid and inotropic drugs via a haemodynamic therapy algorithm may result in a reduction of postoperative complications (Pearse *et al.*, 2014). There have been strategies to ensure the maintenance of perioperative normothermia (Moola & Lockwood, 2011) as well as enhanced recovery programmes (Grace *et al.*, 2011). Not all of these interventions have been as successful. It has long been recognised that patients with a raised preoperative heart rate were at an increased risk of cardiovascular events (Devereaux *et al.*, 2005). As a result randomised controlled trials have been performed to preoperatively beta-blockade those patients. This intervention although reducing the prevalence of cardiovascular events led to increases in the incidence of stroke, death, hypotension and bradycardia (Blessberger *et al.*, 2014; Wijeysundera *et al.*, 2014). However in a high-risk cohort of patients undergoing major abdominal surgery, postoperative infection

occurred close to ten times more frequently than myocardial ischemia or infarction (Pearse *et al.*, 2014). This is clearly an under appreciated postoperative cause of morbidity and mortality.

Following this recognition of the risks associated with elective non-cardiac surgery, particularly in those over 65 years (Jhanji *et al.*, 2008), there have been an increasing number of patients who are routinely admitted to the critical care unit postoperatively (Pearse *et al.*, 2011). This has led to the creation of perioperative physicians, clinicians who are predominantly anaesthetists, who aim to address the complex medical needs of patients during their surgical treatment (Grocott & Pearse, 2012).

Medicine has also begun to translate ideas designed to improve safety and minimise error from areas such as the aviation industry, with the introduction of checklists into clinical practice. This aims to standardise certain aspects of perioperative care across the world ensuring that interventions such as the presence of basic monitoring or if any blood loss is anticipated then adequate access and products are available (Haynes *et al.*, 2009). This checklist was trialled in a diverse group of eight hospitals around the world with implementation of the list demonstrating a reduction in morbidity and mortality (Haynes *et al.*, 2009).

1.4 The Rationale Of This Thesis

As has already been summarised severe polytrauma causes both a shock state and tissue damage. In order to tease out the influence that shock and tissue damage has on the adaptive immune system two patient models have been used:

Major elective abdominal surgery, provides a controlled model of tissue damage, in the absence of shock. This model has advantages such as the availability of preoperative sampling (allowing each patient to act as their own control), predictable sampling timings, preoperative informed consent and a degree of homogeneity.

On the other hand severe polytrauma, used for the patient model of shock and tissue damage, poses a number of challenges. Logistically these patients are difficult to recruit due to their unpredictable admission timings and it can be difficult to gain consent as they often lack capacity in the emergency department. Polytrauma by its nature is a heterogeneous disease that commonly happens to younger fit patients and as a result age appropriate normal healthy controls are used as comparators.

1.5 The Inflammatory Response To Tissue Damage

1.5.1 Introduction

The immune response to major tissue damage has been recently extensively characterised (Lord *et al.*, 2014). Although severe traumatic injury as a model provides information that is highly relevant and applicable to perioperative medicine, the picture is clouded by confounding influences such as hypoperfusion and the subsequent reperfusion injury, the frequent transfusion of allogeneic blood products and neuro-inflammatory mediated changes secondary to an often coexisting traumatic brain injury (Lord *et al.*, 2014). The key link between tissue damage and subsequent inflammation is the release of alarmins (Oppenheim & Yang, 2005).

1.5.2 Alarmins

Alarmins, often termed Damage-Associated Molecular Pattern molecules (DAMPs), are a group of structurally diverse compounds released following tissue damage as cells undergo physiological stress or necrosis (Chan *et al.*, 2012). DAMPs are the endogenous equivalent of Pathogen Associated Molecular Pattern molecules (PAMPs) which initiate an immune response in the setting of infection. Indeed, DAMPs such as high-mobility-group box (HMGB) 1 and mitochondrial DNA (mtDNA) bear significant structural homology to their PAMP analogues and often activate the same pattern recognition receptors (PRRs) (Akira & Takeda, 2004; Chan *et al.*, 2012). This, in part, explains why the clinical pictures of

severe sterile inflammation and sepsis can be difficult or impossible to distinguish. A wide variety of PRRs have been described including the membrane bound Toll-like receptors (TLRs) and the cytoplasmic NOD-like receptors (NLRs) (Chan *et al.*, 2012; Manson *et al.*, 2012). Activation of PRRs induces an enzymatic cascade, which results in down-stream phosphorylation of transcription factors such as NF-κB, which in turn alters cytokine transcription. Immune cell subtypes are activated dependent on their expression of specific PRRs on their cell surface at the time of alarmin release (Manson *et al.*, 2012). Alarmins not only activate this innate response but also provide a vital link between the innate and adaptive immune systems by activating antigen-presenting cells such as monocytes and dendritic cells (Bianchi & Manfredi, 2007).

1.5.3 The Reductionist Approach to Analysis

Until quite recently, studies exploring the inflammatory response to major tissue damage have been mainly limited to a reductionist approach where correlations have been sought between clinical end points and a limited number of candidate mediators. The success of this approach has varied depending on the end point chosen and the assay methodology, with quantitative polymerase chain reaction (qPCR) quantification of messenger RNA (mRNA) transcripts proving more sensitive than enzyme linked immunosorbant assays (ELISA) quantification of protein product. Levels of interleukin 6 (IL-6) and IL-10 consistently rise in proportion to the extent of the tissue damage and levels are associated with a greater incidence of subsequent nosocomial infection (Baigrie *et al.*, 1992; Fragkou *et al.*, 2014; Giannoudis *et al.*, 2000; Mokart *et al.*, 2005b). As arguably the most potent anti-inflammatory cytokine it is unsurprising that high IL-10 levels are associated with later infection. However, although IL-6 is traditionally considered a pro-inflammatory cytokine it also up-regulates suppressor of cytokine signalling (SOCS)-1 expression and inhibits T helper cell type 1 (T_h1) differentiation (Diehl & Rincon, 2002). In this manner, IL-6 could plausibly exert an effect that limits effective host bactericidal capacity. Similarly, the expression of Human Leukocyte Antigen DR on the surface of monocytes (mHLA-DR) consistently falls

following tissue damage and is related to nosocomial infection (Cheron *et al.*, 2010; Wakefield *et al.*, 1993). Again this is unsurprising as the maintenance of adequate Major Histocompatibility Complex (MHC) class II molecules such as HLA-DR on the surface of antigen presenting cells is crucial to maintain immune competence. Others have proposed genomic signatures where higher ratios of anti-inflammatory to pro-inflammatory cytokines correlate with postoperative infection (White *et al.*, 2011). What remains elusive is the mechanism whereby alarmin release and the subsequent enzymatic cascades lead to an immunosuppressed phenotype and the survival advantage, if any, of this trait.

1.5.4 The Proposed Genomic Storm

Advances in technology recently permitted the simultaneous analysis of the leukocyte transcriptome of 20,720 genes in patients following severe blunt trauma and burn injury in a landmark paper (Xiao *et al.*, 2011). Following these stimuli, which would clearly result in significant alarmin release, 80% of cellular pathways and functions were altered. Innate immunity pathways, B-cell receptor signalling and IL-10 signalling all demonstrated up-regulated gene expression whereas antigen presentation and T-cell activation were down-regulated. Importantly, it was the overall magnitude of the genomic alterations which correlated with nosocomial infections and organ impairment as opposed to differential activation of specific pathways. Although this snapshot of the transcriptome was within 12 hours of injury, in some cases patients had undergone extensive resuscitation and therefore this heterogeneous picture should be interpreted with caution in the context of the perioperative patient. This paper has however helped to re-define the previously proposed bimodal inflammatory response model to tissue damage, suggesting concomitant activation of pro-inflammatory and anti-inflammatory pathways.

1.5.5 Mass Cytometry & Further Single-Cell Analysis Developments

Further application of advanced technology has seen investigators utilise mass cytometry to detect surgery-induced immune perturbations in clinical samples and relate

these findings to postoperative recovery (Gaudilliere *et al.*, 2014). Mass cytometry involves using antibodies to tag cellular components prior to nebulising the cells and then using a time of flight mass spectrometer for analysis. This complex technique has extensively described the immune response in peripheral blood following elective hip arthroplasty and has demonstrated a time-dependent and cell type specific activation of immune signalling networks.

Over the early postoperative period there is an expansion of Natural Killer (NK) cells, neutrophils and $CD14^+$ monocytes, which is followed, within 24 hours, by contraction of $CD4^+$ and $CD8^+$ T cells. Most notable was a six-fold expansion of $CD33^+CD11b^+CD14^+HLA\text{-}DR^{low}$ monocytes with phenotypic similarities to myeloid derived suppressor cells (MDSCs). MDSCs are a heterogeneous group of immunosuppressive cells (Gabrilovich *et al.*, 2007) which remain poorly defined in terms of cell surface markers and directly suppress T cell functions through a variety of mechanisms including the production of reactive oxygen species (ROS) (Schmielau & Finn, 2001) and Arginase-1 (Highfill *et al.*, 2010) as well as IL-10 and TGF-β release (Ostrand-Rosenberg & Sinha, 2009). In addition to the monocyte derived MDSCs that expand following hip arthroplasty, a distinct subset of $CD62L^{dim}$ neutrophil-derived MDSCs appear shortly after blunt trauma and tissue injury and induce T cell suppression in a Mac-1 (CD11b) dependent fashion (Pillay *et al.*, 2012; Pillay *et al.*, 2013). Further characterisation of the expansion of MDSCs in the postoperative period may provide a vital link between alarmin release and an immunosuppressed phenotype. An analysis of surgery induced changes in the phosphorylation of intracellular signalling proteins in different immune subsets provides interesting correlations, particularly in the $CD14^+HLA\text{-}DR^{low}$ monocyte clusters where immune correlates, such as STAT3 signalling, account for up to 60% of the variability in postoperative recovery (Gaudilliere *et al.*, 2014). It is particularly relevant that in another cohort preoperative differences in monocyte STAT signalling pathways correlate to postoperative complications (Lahiri *et al.*, 2015).

1.5.6 Circulating Cellular Aspects Of The Immune Response

1.5.6.1 Introduction

The majority of circulating cells of the immune system are referred to as leukocytes and have in the past been broadly divided into two categories, those influencing the innate or the adaptive arms of the immune system. The innate immune has traditionally been thought to provide an acute but less specific immune response, while the adaptive arm provides a more specific but delayed immune response that is antigen mediated (Janeway, 2012). These terminologies are perhaps now becoming archaic with more recent single cell analysis demonstrating $CD4^+$ STAT3 and STAT5 upregulation only an hour following hip arthroplasty (Gaudilliere *et al.*, 2014).

1.5.6.2 Neutrophils

Neutrophils are granulocytes and in humans are the most common circulating leukocyte and are elevated following infection or following tissue damage. They, along with monocytes characterise the immediate immune response (Leliefeld *et al.*, 2015) assisting in the clearance of infection and debris. Previously, they were thought to be a short-lived and homogenous population. However recent evidence suggests that they are able to survive in the circulation for an average of 5.4 days (Pillay *et al.*, 2010), this was at least 10 times longer than had previously been appreciated. The same group has also reported a novel heterogeneity of neutrophils, with a mature form of neutrophils phenotyped as $CD11c^{bright}/CD62L^{dim}/CD11b^{bright}/CD16^{bright}$ showing immunosuppressive actions on circulating T cells (Pillay *et al.*, 2012). Neutrophils are classically regarded as part of the innate immune system, displaying a number of receptors on their cell surface (pattern recognition and F_c receptors amongst others) these features allow them to respond to a wide variety of stimuli (Leliefeld *et al.*, 2015). They not only secrete cytokines to signal to other cell types but can also destroy bacteria and fungi either by phagocytosing them or via the production of reactive oxygen species (ROS) or Neutrophil Extracellular Traps (NETs) (Branzk *et al.*, 2014; Brinkmann *et al.*, 2004; Leliefeld *et al.*, 2015). Neutrophils are able to

move into the tissues from blood vessels a process known as diapedesis, in order to migrate into inflamed tissue. What has recently been described is the ability of neutrophils to reverse transmigrate, moving from the tissues across the endothelium into the bloodstream, possibly further potentiating an inflammatory response (Woodfin *et al.*, 2011).

1.5.6.3 Monocytes

Monocytes are another form of granulocyte and they are also able to phagocytose, thus assisting in the clearance of infection and debris (Gordon & Taylor, 2005). While circulating, they can be characterised into three distinct subsets based on the expression of CD14 and CD16 (Gordon & Taylor, 2005), with the most prominent of the subsets, classical monocytes, phenotyped as $CD14^{++}CD16^{-}$. This subset is known to produce high levels of pro-inflammatory cytokines following stimulation by endotoxin (Wong *et al.*, 2011). Intermediate monocytes, phenotyped as $CD14^{+}CD16^{+}$, are high expressers of HLA-DR and as a consequence play a key role in presenting antigens to T cells. The final subset, non-classical monocytes, are the least common and are phenotyped as $CD14^{dim}CD16^{++}$and these cells are primarily involved in the response to viral infections (Alazawi *et al.*, 2016). Monocytes differentiate when in the tissues to macrophages or myeloid dendritic cells (mDCs).

As has already been discussed earlier in this introductory Chapter there is a great deal of interest in the biology of MDSCs especially in the context of cancer, sepsis and tissue damage. At the moment, in humans, these cells are poorly phenotyped as there is a mixed and unclear heritage to these cells. This is summarised in an excellent review, which outlines our current understanding of the characteristics of the myeloid-derived and the monocyte-derived suppressor cells (Pillay *et al.*, 2013).

Other granulocytes such as Eosinophils, Basophils and Mast cells are less common in the circulating blood. Eosinophils are primarily involved in the response to parasites (Kita, 2011), while Basophils and Mast cells are involved in allergic reactions, both being potent producers of histamine (Schroeder, 2011).

1.5.6.4 Dendritic Cells

Dendritic cells are the circulating antigen presenting cells and as a consequence they act as a vital link between the innate and adaptive immune system, determining the magnitude and quality of the adaptive immune response (Boltjes & van Wijk, 2014). These cells are broadly classified into two differing types based on their heritage; conventional dendritic cells, previously termed myeloid dendritic cells (two differing forms phenotyped as $CD1c^+$ or $CD141^+$), are potent secretors of the pro-inflammatory cytokine IL-12 while plasmacytoid dendritic cells (pDCs, $CD303^+$) secrete interferon-α (Boltjes & van Wijk, 2014). Sterile or non-sterile inflammation is known to affect the function of dendritic cells causing increased rates of apoptosis as well as reductions in antigen presentation capability and cytokine production capacity, thus impeding the later activation of the adaptive immune response (Hotchkiss *et al.*, 2013).

1.5.6.5 Lymphocytes

Lymphocytes were previously thought to be exclusively adaptive immune cells, however a family of innate lymphoid cells (ILCs) have being recently better characterised. Although these cells occupy a small percentage of the total circulating lymphocyte count they do not exhibit any degree of antigen specificity allowing them to act rapidly and influence the impending adaptive immune response (Eberl *et al.*, 2015; Klose & Artis, 2016).

The remainder of the lymphocyte population can be divided into T (Thymus) or B (Bone marrow) derived cells. T cells can further be divided in those expressing CD4 or CD8 cell surface markers, dividing them into Helper or Cytotoxic cells. $CD4^+$ (Helper) T cells are the subpopulation predominantly discussed (in greater detail later) in this thesis. Initially these cells were thought to be divided into T_h1 or T_h2 populations. With T_h1 populations acting primarily as host immunity effectors against bacteria and protozoa, while T_h2 populations acted against parasites (Abbas *et al.*, 1996). Further subsets of T helper cells have since been discovered including T_h17, T_h22 and follicular cells meaning this previously proposed reductionist model of T_h1 or T_h2 polarisation theory has largely been abandoned

(Nakayamada *et al.*, 2012). T cell receptor signalling has been shown to be downregulated both following trauma (Xiao *et al.*, 2011) and sepsis (Boomer *et al.*, 2012). These cells also exhibit signs of exhaustion via the expression of cell surface markers such as Programmed cell Death protein (PD-1) or Cytotoxic T-lymphocyte-Associated Protein 4 (CTLA-4) following tissue damage or infection, in a similar manner to what is exhibited in cancer biology (Hotchkiss & Moldawer, 2014). T regulatory cells (Tregs) are also $CD4^+$ and are involved in the maintenance of immune tolerance. Following sepsis, trauma or surgery these are thought to be increased (Cavassani *et al.*, 2010), contributing to the anti-inflammatory environment by inhibiting neutrophil and monocyte function (Hotchkiss *et al.*, 2013; Venet *et al.*, 2006). $CD8^+$ cells are commonly referred to as Cytotoxic T cells. These are typically involved in the response to cancer, viral infections or following transplantation; these also are thought to undergo apoptosis following tissue damage or infection, thus also impacting upon the body's defense mechanism against intracellular infections with certain viral, protozoan, and bacterial pathogens (Harty *et al.*, 2000).

All of these T cells can be broken down into effector, memory or naïve cells. Effector cells will actively respond to co-stimulation, while memory cells are specific cells that are retained following an infection, and as a result are able to expand at short notice. Naïve T cells are cells awaiting differentiation following antigen presentation (Janeway, 2012).

B ($CD19^+$) cells play a dual role in the inflammatory response. They are not only able to present antigens to T cells but are able to produce antibodies (Vaughan *et al.*, 2011). Following sepsis the rate of apoptosis is thought to increase, alongside their ability to produce antigen-specific antibodies (Hotchkiss *et al.*, 2001). Following tissue damage their role is poorly defined, however more recently gene array data described in the genomic storm paper suggests that there was unregulation of B cell signalling following trauma (Xiao *et al.*, 2011).

1.5.6.6 Platelets

Although platelets have been long regarded as predominantly a component of the coagulation system their role in shaping the immune response is only more recently being appreciated (Morrell *et al.*, 2014). Platelets are able to contribute to the immune response by forming immunocomplexes with circulating leukocytes thereby influencing their cytokine secretion pattern as well as secreting their own cytokines such as IL-1β, thus contributing to the acute phase response (Lindemann *et al.*, 2001). Platelets are also able to expel granules and microparticles further influencing the trajectory of the immune response (Morrell *et al.*, 2014).

1.5.7 Conclusions

These data demonstrate that our understanding of the immune response to surgery and tissue damage is rapidly expanding in conjunction with available technology and provides opportunities for the identification of therapeutic targets and predictive biomarkers.

1.6 Anaesthesia, Analgesics & The Inflammatory Response

1.6.1 Introduction

Whilst the presence of significant tissue damage exerts the dominant influence on altered perioperative immunity, the administration of anaesthetic agents has additional and complex effects. In the clinical scenario it can be very difficult confidently to separate the immune modulating effects of anaesthesia from the response to surgery and tissue damage and consequently much of the available mechanistic data is generated either from *in vitro* experimental work or animal models.

1.6.2 General Anaesthesia & Inflammation

Broadly speaking, the overriding effect of anaesthesia on the immune system is one of suppression and is mediated both directly and indirectly. Inhalational and intravenous

anaesthetics induce lymphocyte apoptosis and impair neutrophil phagocytosis (Matsuoka *et al.*, 2001). Secondary immunosuppressive effects are mediated through modulation of the neural immune-regulatory circuit and activation of cholinergic anti-inflammatory pathways and also as a consequence of altered adrenocortical functions (Picq *et al.*, 2013). Opioids are administered frequently during anaesthesia and their inhibition of innate and adaptive immunity is well described (Vallejo *et al.*, 2004). Natural killer cells, a key facet of innate immunity and host tumour surveillance, are suppressed by both anaesthesia and opioids (Markovic *et al.*, 1993). Clearly, the choice of anaesthetic technique may have important clinical implications independent of the surgical procedure. The presence of an anaesthesia-induced immunocompromised phenotype may affect outcome in different ways but in the perioperative setting it is the creation of a pro-tumour and pro-infection cytokine and inflammatory milieu that is of key concern. Cancer and infection are intimately linked as both flourish in an environment of T cell exhaustion and lymphocyte anergy, such as is observed in the perioperative period (Hotchkiss & Moldawer, 2014). It is also notable that both conditions themselves also induce this phenotype, which has additional implications for those patients with chronic infections and malignancies who undergo operative treatment.

1.6.3 Neuraxial Anaesthesia

Although the hormonal stress response is not completely ablated by the use of regional anaesthesia, avoiding general anaesthesia is associated with a blunted response and lower peak levels of serum cortisol (Milosavljevic *et al.*, 2014). Interestingly, in a large cohort of patients undergoing knee arthroplasty the administration of neuraxial anaesthesia alone when compared to general anaesthesia alone was associated with a decreased incidence of postoperative infections (Liu *et al.*, 2013a). The odds of pneumonia occurrence were 0.51 in those patients receiving neuraxial anaesthesia alone when compared to general anaesthesia alone in this cohort. Whilst this study was not randomised it is important that the association remained following a propensity-matched analysis.

1.6.4 Conclusions

A strategy of limiting, but not excluding, inhalational and intravenous anaesthetics by combining epidural and general anaesthesia has suggested subtle advantages over general anaesthesia alone in terms of the duration of postoperative immunosuppression, reduction in absolute T lymphocyte count and the relative proportions of T_h1, T_h2 and T_{reg} cell subsets (Chen *et al.*, 2015; Cheng *et al.*, 2013). However, the clinical benefit of this combined approach remains unclear. A meta-analysis of studies comparing a technique of combined epidural and general anaesthesia versus general anaesthesia alone failed to demonstrate convincingly a benefit in terms of cancer recurrence (Pei *et al.*, 2014). However, in a review of nearly 400,000 patients undergoing hip or knee arthroplasty, whilst the benefit of neuraxial anaesthesia alone was replicated in terms of lesser infection risk any protective effect appeared markedly reduced in the cohort that received a combined general and regional anaesthesia technique (Memtsoudis *et al.*, 2013).

In each of the above studies the absence of randomisation makes interpretation difficult. Consequently, these studies are prone to inherent biases making it impossible to draw definitive conclusions and they should be viewed as hypothesis generating. This viewpoint is supported by a recent consensus statement expressing concern that experimental evidence suggests a link between anaesthetic technique and cancer recurrence yet accepts that there is insufficient clinical evidence to justify any change in practice and calls for the conduct of definitive randomised clinical trials (Buggy *et al.*, 2015).

1.7 Common Adjunctive Perioperative Treatments & Inflammation

1.7.1 Dexamethasone & Other Commonly Administered Perioperative Therapeutics

Dexamethasone is frequently administered during anaesthesia as an effective prophylactic anti-emetic. Single doses have additional beneficial effects such as enhanced analgesia and reduced surgical site swelling. However, it is a potent glucocorticoid and even single doses can display effects on adrenocortical functions a number of days following administration (Elston *et al.*, 2013). Although there is clear physiological rationale for implicating dexamethasone in enhancing the risk of postoperative infection the clinical data have been conflicting (Bolac *et al.*, 2013; Corcoran *et al.*, 2010; Percival *et al.*, 2010). A recent meta-analysis of randomised controlled trials using single-dose dexamethasone found no association with postoperative infection (Waldron *et al.*, 2013). These results should however be interpreted cautiously as the dexamethasone group also received less opioids, thereby introducing a potential source of bias.

Other ubiquitously prescribed perioperative treatments with potential immunomodulating properties, such as paracetamol, NSAIDs and gabapentinoids, have not demonstrated clear associations with important immune outcomes such as infection (Mathiesen *et al.*, 2014).

1.7.2 Allogeneic Blood Transfusion

The immunomodulating qualities of perioperative allogeneic blood transfusion have long been appreciated and have even been exploited to prevent renal allograft rejection in the era prior to the development of effective immunosuppressants (Opelz & Terasaki, 1978). The unintended clinical consequences of perioperative immune modulation by allogeneic blood, particularly following colorectal surgery, include an increased susceptibility to infectious

complications. It is not suggested that the transfused products contain pathogenic microbes; rather the immune response induced by the transfusion creates an environment conducive to the growth of pathogenic organisms. Allogeneic transfusion has also been implicated in the recurrence of cancer following general surgical procedures (Amato & Pescatori, 2006; Cata *et al.*, 2013; Jensen *et al.*, 1992). However, more recently similar links between transfusion and cancer recurrence have also been reported following surgery for prostate, hepatic, and head-and-neck cancers (Danan *et al.*, 2015; Schiergens *et al.*, 2015; Soubra *et al.*, 2014).

An autologous blood transfusion, used more frequently in cardiac and orthopaedic surgery, is potentially associated with less severe immune alterations when compared with allogeneic blood transfusion. Supporting this are data from over 12,000 patients undergoing elective hip or knee arthroplasty where patients receiving an autologous blood transfusion were less likely to suffer respiratory tract infections or wound infections (Friedman *et al.*, 2014).

Interestingly, blood stored for prolonged periods prior to administration may be particularly deleterious with *in vivo* models suggesting that aged red blood cells may exert enhanced tumour progression (Atzil *et al.*, 2008). However, recently, two multi-centre RCTs assessing the influence of the duration of storage of PRBCs prior to transfusion failed to demonstrate associations between 'older' PRBCs and the development of nosocomial infections in septic patients (Lacroix *et al.*, 2015) or in patients undergoing cardiac surgery (Steiner *et al.*, 2015). Despite this, in a recent meta-analysis, when looking specifically at trauma and cardiac surgery patient groups those receiving 'older' PRBCs may be more susceptible to nosocomial infections (Ng *et al.*, 2015).

1.8 Epigenetics & Immune Responsiveness

1.8.1 Introduction

Epigenetics is an umbrella term that describes host mechanisms of altering gene expression that do not require a change in the underlying DNA sequence. The enzymatic conversion of cytosine to 5-methylcytosine and the methylation or acetylation of chromatin usually causes transcriptional repression by impeding access to promoter regions whereas the overexpression of micro RNAs (miRs) can both inhibit transcription and target messenger RNA (mRNA) for degradation. Cancer research has pioneered the study of epigenetic modifications that promote an immunosuppressed phenotype, thereby facilitating immune evasion by cancerous cells, and has also been at the forefront of developing epigenetic modifying agents that can target these processes (Héninger *et al.*, 2015).

1.8.2 Epigenetics & Immunomodulation

In the perioperative period epigenetic studies have largely focused on acute and chronic pain processes, although data supportive of a key role in inflammation and immunosuppression have emerged (Lirk *et al.*, 2014). For example, the use of opioids in the perioperative period promotes global DNA methylation in peripheral blood leukocytes (Doehring *et al.*, 2013). This is consistent with the transcriptional repression of pro-inflammatory genes, which may have longer-term implications as epigenetic alterations persist. Furthermore, our group have described the post-traumatic production of miRNAs with sequence complementarity to the mRNA transcripts of key cytokines whose expression levels change markedly following tissue damage (Owen *et al.*, 2014). This may represent an epigenetic regulation of the response to tissue damage through the targeted degradation of pro-inflammatory mRNAs by miRs. In this setting miR levels also correlate with nosocomial pneumonia. Although the study of epigenetics in the perioperative period is in its infancy the therapeutic and diagnostic implications may be substantial.

1.9 Conclusions

Rapid advances in our understanding of perioperative inflammatory processes, their causes and consequences coincide with development of multiple, clinically applicable immune and epigenetic modulators such as growth factors, antibodies, DNA hypomethylating agents, histone deacetylase inhibitors and micro RNA mimics. The prospect of manipulating an errant immune response to major surgery is no longer aspirational. Personalised medicine has become a reality for many patients suffering from a variety of immune related disorders such as myelodysplasia, rheumatoid arthritis and inflammatory bowel disease. These patients now routinely benefit from therapies which target specific facets of a pathological immune response and the challenge for perioperative medicine is to distinguish between protective and pathogenic immune responses in the perioperative period and to identify modifiable immune pathways which when altered can impact on important clinical endpoints.

Uniquely, the elective nature of the majority of surgical procedures introduces the possibility of developing a pre-emptive, preventative, immunotherapy strategy that may ultimately prove advantageous. The potential for pre-emptive or early therapies for those undergoing scheduled procedures vastly increases the prospects of success for any intervention for the perioperative patient. To achieve this ultimate aim basic scientists must continue to define pathological inflammatory pathways and collaborate with translational scientists identifying interventions suitable for clinical use. Clinical trialists must also be engaged with this process so that potential patient benefits are revealed in well-designed clinical trials.

1.10 Aims Of This Thesis

- To document changes in the expression of genes that describe T Helper cell inflammatory pathways in polytrauma and major abdominal surgery.

- To describe associations between patterns of T-Helper cell related gene expression and key clinical outcomes.

- To define the influence of allogeneic blood transfusion on these pathways as well as the impact that transfusion has on the acquisition of nosocomial infection.

- To identify soluble mediators that circulate following tissue damage that impart a detrimental immunomodulatory effect and to describe how these effects may be reversed.

Chapter Two: Methods

2.1 Activation of Coagulation & Inflammation in Trauma 2 (ACIT2)

The ACIT2 study commenced recruiting at the Royal London Hospital in January 2008 having received ethical approval from the East London and City Regional Ethics committee 1 on 13th November 2007 (07/Q0603/29). Initially it was developed as a stand-alone observational study aiming better to characterise the initial coagulopathy findings of Professor Karim Brohi. This was later extended to encompass inflammatory changes, increasing the volume of blood that was ethically permitted to be drawn, allowing the collection of greater volumes of plasma for ELISA analysis as well as the storage of PAXgene® tubes for the analysis of whole blood total RNA. To collect these data there is a dedicated rota of fellows on-call from 08:00 – 20:00 (later extended to 22:00) who screen all trauma team activations (Supplementary Table 1) for eligible candidates. All those patients screened are recorded for ethical purposes.

2.1.1 Inclusion & Exclusion Criteria

All trauma patients presenting to the Royal London Hospital are eligible for recruitment to ACIT2. However, pragmatically, latterly those less severely injured patients who are likely to be discharged < 24 hours are not recruited. The exclusion criteria are:

- Age <16 years
- Transfer from another hospital
- Arrival > 120 minutes from injury
- More than 2000ml crystalloid pre-hospital
- More than 5% total body surface area (TBSA) burns
- Severe liver disease

- Known bleeding abnormality (including anticoagulant medication)
- Refused consent
- Vulnerable patients (in custody or in prison)

For the purposes of this thesis those diagnosed with infection with the human immunodeficiency virus, or with neutropenia as a result of chemotherapy or receiving long-term treatment with corticosteroids were also excluded. Only patients admitted initially to the Adult Critical Care Unit following resuscitation and/or damage control surgery were included in the analysis.

2.1.2 Consent

On admission, it is frequently impossible and often unethical to attempt to obtain informed consent from the patient or their next of kin. To address this, emergency research permits the use of a professional legally appointed representative in accordance with the Mental Capacity Act 2005 (Great Britain. Department for Constitutional Affairs., 2007). The professional legally appointed representative (LAR) is the trauma team leader (usually a consultant or senior registrar in the Emergency Department, who has undergone good clinical practice (GCP) training), who has been briefed on the study protocol and therefore is able to provide independent assent. Following professional LAR consent the research team are then able draw 30mL of blood on admission and at the two following sampling points (24 and 72 hours), if the patient is still lacking capacity. Every effort is made following admission to seek patient consent or LAR assent from relatives as soon as possible. If consent is then refused then samples and the related data are destroyed. Entry into the study did not influence clinical treatment.

2.1.3 Blood Collection

As previously described blood sampling takes place at 3 time points, on admission to the Emergency Department, at 24 and 72 hours. As the majority of blood is used for coagulation studies only the relevant sampling tubes used for this thesis will be discussed.

- 2 x 4.5 mL citrated vacutainers for plasma collection (0.109M + buffered sodium citrate 3.2%, Becton Dickinson, Plymouth, UK).

- 1 x 2.5 mL RNA PAXgene® for genetic analysis (PreAnalytix, Germany).

- A paired standard panel of blood tests for routine laboratory processing including; Full blood count, coagulation screen, fibrinogen and D-dimer.

- A blood gas (BG) is also drawn as part of standard care at admission and at 24h or 72h if patients have a central or arterial line in-situ.

2.1.4 Processing & Storage of Samples

2.1.4.1 Citrated plasma (ACIT)

The citrated vacutainer tubes were centrifuged at 3400 RPM for 10 minutes, with the brake on, in a Clinispin Horizon 853VES Laboratory Centrifuge (Woodley Equipment Company Ltd, UK). The resulting sample then separated into three layers; citrated plasma, buffy coat and erythrocytes. The top two thirds of the plasma from each tube was aliquoted into a clean 5 mL Falcon tube (Becton Dickinson, UK) using a Pasteur pipette and centrifuged for a second time at 3400 RPM. The double-spun, platelet-poor plasma was then transferred, using a Pasteur pipette, in 500 µL aliquots, to 3 x 0.6 mL Cliklok microcentrifuge tubes (MCT) (Simport, UK). The lower third of the plasma from each tube was transferred, in 500 µL aliquots, to a 0.6 mL Cliklok microcentrifuge tube (Simport, UK) for storage as single spun plasma. The buffy coat was then aliquoted using a Pasteur pipette, attempting to minimize red-cell contamination, into a 1.5 mL microcentrifuge tube (Fisher Scientific, UK).

The remainder of the sample was then discarded. All samples were labelled and stored in catalogued cryoboxes at -80°C immediately following processing.

2.1.4.2 PAXgene®

PAXgene® tubes were inverted 10 times and stood upright on the lab bench for a minimum period of two hours. Following this they were stored upright at -20°C for a minimum of 24 hours before being transferred to the -80°C freezer for long-term storage.

2.1.4.3 Blood Gas

Blood gas analysis was performed using a near patient testing device, ABL 500 Flex (Diamond Diagnostics, USA) within the ED department or the Adult Critical Care Unit. All of these machines are calibrated every four hours.

2.1.5 Data collection

Extensive data was collected characterising the; injury severity (Injury Severity Score (ISS) & Abbreviated Injury Score (AIS)), shock state (blood gas and haemodynamic variables) as well as the prevalence of nosocomial infection (Horan *et al.*, 2008) and organ dysfunction (measured by the Sequential Organ Failure Assessment (SOFA) score (Vincent *et al.*, 1996)) for those patients in the adult critical care unit (ACCU) and for all patients recruited into this study. The trauma research fellows collected these data prospectively on a day-to-day basis.

2.1.5.1 Scoring Systems

2.1.5.2 ISS

The Injury Severity Score (ISS) was the first score developed to grade the severity of injuries and their relationship with mortality (Baker *et al.*, 1974). The score is calculated retrospectively following the identification of all injuries to the patient. Each anatomical region is graded between 0 and 5 to obtain an abbreviated injury score (AIS). The three highest AIS scores are then squared and combined to form a total from a maximum of 75.

Scores ≥16 are regarded as major trauma, whilst those ≥25 are severe trauma. This scoring system is further outlined in Supplementary Table 2.

2.1.5.3 The Sequential Organ Failure Assessment (SOFA) Score

Internationally in trauma research there is no widely agreed consensus as to the best way to score and diagnose organ dysfunction. A number of scoring systems have been devised to describe the severity of organ failure consistently between patients but selection remains centre specific. The SOFA, Marshall Score and Denver Score are three of the most widely used tools. The parameters used in this scoring system are further outlined in Supplementary Table 3.

In this thesis the development of organ dysfunction was assessed daily using the Sequential Organ Failure Assessment (SOFA) score (Vincent *et al.*, 1996). Single-organ dysfunction was defined as a SOFA score of ≥3 in one organ system during any 24-hour period for the duration of the patient's ICU stay. Multiple-organ dysfunction was defined as a SOFA score of ≥3 in ≥2 organ systems during any 24-hour period (Moreno *et al.*, 1999). Acute Adult respiratory distress syndrome (ARDS) was deemed to be present when the respiratory component of the SOFA score was ≥3 for 2 consecutive 24 hour periods, the limitations of this are discussed in the conclusion of this thesis.

2.2 Vascular Events In Noncardiac Surgery Patients Cohort Evaluation (VISION)

The VISION trial, a multicentre international observational study, commenced recruiting at the Royal London Hospital in March 2011 having received ethical approval from the North Wales Research Ethics Committee (Reference: 10/WNo03/25). The cohort of patients that was included in this study was recruited from June 2012 to November 2013. The

VISION study aimed to describe the relationship between the peak fourth-generation troponin T measurement over the first 3 days following elective non-cardiac surgical patients (over the age of 45) and 30-day mortality (Vascular Events In Noncardiac Surgery Patients Cohort Evaluation Study *et al.*, 2012). Sampling took place, preoperatively, and at 24, 48 and 72 hours postoperatively. Patients gave consent, and bloods were drawn by the critical care research team. Patients were free to withdraw their consent at any point or refuse blood sampling.

2.2.1 Inclusion & Exclusion Criteria

All patients aged 45 or over undergoing scheduled surgery at the Royal London Hospital involving the gastrointestinal tract, requiring a general anaesthetic and at least an overnight hospital stay were eligible for recruitment to VISION. The exclusion criteria were:

- Age <45
- Refusal of consent
- Emergency surgery
- Surgery, which also involved access to the thoracic cavity

2.2.2 Consent

Every patient on a weekday elective operating list was screened. Eligible patients were approached for written informed consent by a member of the research team. All team members had undergone good clinical practice (GCP) training. Entry into the study did not influence clinical treatment.

2.2.3 Blood Collection

As previously described blood sampling takes place at 4 time points, preoperatively, and at 6-12, 24, 48 and 72 hours postoperatively. In addition to serum, which was drawn for the analysis of troponin levels, a number of other vials were collected.

- 1 x 4.5 mL citrated vacutainers for plasma collection (0.109M + buffered sodium citrate 3.2%, Becton Dickinson, Plymouth, UK)
- 1x 5.0 mL SST^{TM} II advance vacutainer (Becton Dickinson, Plymouth, UK)
- 1 x 2.5 mL RNA PAXgene® for genetic analysis (PreAnalytix, Germany)

2.2.4 Processing & Storage of Samples

2.2.4.1 Citrated plasma

The citrated vacutainer tubes were centrifuged at 3000 RPM for 10 minutes, with the brake on, in a Sigma 2-16PK centrifuge (Sigma, UK). The resulting plasma was then aliquoted into 2.0 mL Corning® cryogenic vials (Corning Incorporated, NY, USA). All samples were labelled and stored in catalogued cryoboxes at -80°C immediately following processing.

2.2.4.2 Serum

The SST^{TM} II vacutainer tubes were centrifuged at 3000 RPM for 10 minutes, with the brake on, in a Sigma 2-16PK centrifuge (Sigma, UK). The resulting serum was then aliquoted into 2.0 mL Corning® cryogenic vials (Corning Incorporated, NY, USA). All samples were labelled and stored in catalogued cryoboxes at -80°C immediately following processing.

2.2.4.3 PAXgene®

PAXgene® tubes were inverted 10 times and stood upright on the lab bench for a minimum period of two hours. Following this they were stored upright at -20°C for a minimum of 24 hours before being transferred into the -80°C freezer for long-term storage.

2.2.5 Data collection

Patients were examined daily by the clinical team for the presence of infection. Definitions of infection were agreed prospectively by the investigators and were based on the Center for Disease Control and Prevention (CDC) definitions (Horan *et al.*, 2008) and graded using the Clavien-Dindo classification (Supplementary Table 4).

2.3 Experimental Methodology

2.3.1 Total RNA Isolation from PAXgene® tubes

2.3.1.1 PAXgene®

PAXgene® tubes are a widely used consumable for the collection of total RNA from whole blood. Due to their ease of use they are ideally suited to large national or international studies allowing a standardised total RNA collection method to be employed.

Prior to starting the extraction, protocol wash buffer 2 (BR4), which is supplied as a concentrate, is made up with ethanol (96–100%, purity grade) as indicated on the bottle to obtain the working solution (BR4).

In addition to this the DNase I stock solution is prepared when the kit is first opened. The solid DNase I (1500 Kunitz units) is dissolved in 550 µL of the DNase resuspension buffer (DRB) provided with the kit. This is then aliquoted into 130 µL volumes (to prevent freeze-thaw cycles) and frozen to -20°C until it is ready for use.

The PAXgene® tubes were removed from the -80°C freezer in batches of twelve the evening prior to processing to allow them to thaw adequately, whilst standing on the laboratory bench. In the morning the twelve tubes were centrifuged for 10 minutes at 3,000 x g (RCF) using a swing-out rotor (4K15 Bench-top Centrifuge, Sigma, UK). The supernatant was carefully decanted, so as not to disturb the pellet and 4 mL of RNase-free water (RNFW) was added to the pellet. The tube was closed using a fresh Hemogard stopper.

The pellet was then re-suspended with a vortex (SA7 Vortex Mixer, Stuart, UK) and centrifuged for 10 minutes using the same settings. Again the entire supernatant was carefully removed, as incomplete removal in this step can inhibit lysis in the following steps and therefore the binding of RNA to the membranes of the columns.

Following this, 350 mL of re-suspension buffer (BR1) was then added to the pellet and this was vortexed until the pellet was visibly suspended. The solution was aliquoted into a 1.5 mL microcentrifuge tube (MCT) and 300 µL of binding buffer (BR2) and 40 µL proteinase K (PK) were added. This was vortexed for 5 seconds, and incubated for 10 minutes at 55°C (having been pre-heated) using a shaker–incubator at 1000 rpm (Eppendorf, UK).

After this incubation, the temperature of the shaker–incubator was altered to 65°C for a later step and all pipettes were treated with RNAse to prevent contamination of the samples. A total of 700 µL of the lysate from the MCT was pipetted directly into a PAXgene® Shredder spin column which was placed in a 2 mL processing tube (PT), and centrifuge for 3 minutes at 14,000 x g (1-15 Mini-centrifuge, Sigma, UK).

The supernatant of the flow-through fraction is transferred into a fresh 1.5 mL MCT without disturbing the pellet in the processing tube. 350 µL of ethanol (96–100%, purity grade p.a.) was added to this MCT and then mixed by vortexing. The MCT was centrifuged briefly (1–2 seconds at 500–1000 x g) to remove drops from the inside of the tube lid. The

length of the centrifuging did not exceed 1–2 seconds, to prevent pelleting of the nucleic acids, thus potentially reducing yields of total RNA.

A 700 µL aliquot of the sample was then pipetted into the PAXgene® RNA spin column, placed in a 2 mL PT. This was then centrifuged for 1 minute at 12,000 x g. The Spin column was then placed in a new 2 mL PT and the flow through was discarded. The remainder of the sample was pipetted into the PAXgene® RNA spin column, and re-centrifuged for 1 minute using the same settings. As before the PT containing flow through was discarded and the PAXgene® RNA spin column was placed in a fresh PT. 350 µL of wash buffer 1 (BR3) was then added to the PAXgene® RNA spin columns and centrifuged for 1 minute at 12,000 x g. The PTs containing the flow through were discarded and the spin columns were re-housed in fresh 2 mL PTs.

A 10 µL volume of DNase I (RNFD) solution, pre-made up and stored at -20°C in batches of 13 aliquots, as previously described, was gently thawed on ice and combined with 70 µL of DNA digestion buffer (RDD). For the processing of batches, 12 samples (plus 1 for pipetting error), consisting of a master mix of 130 µL of RNFD and 910 mL of RDD, was added a 1.5 mL MCT. This was gently mixed by flicking the tube. The RNFD solution is particularly sensitive to physical denaturation so vortexing was not carried out. 80 µL of the master mix was aliquoted, directly onto each of the PAXgene® RNA spin column membranes. These were then rested on the benchtop at room temperature for 15 minutes.

Following the 15 minute incubation 350 µL of wash buffer 1 (BR3) was aliquoted into each of the PAXgene® RNA spin columns. They were re-centrifuged for 1 minute at 12,000 x g. As before, the PTs containing the flow through were discarded and the spin columns were re-housed in fresh 2 mL PTs.

The PAXgene® RNA spin columns were further washed with 500 µL of wash buffer 2 (BR4) and centrifuged for 1 minute at 12,000 x g. As before, the PTs containing the flow through were discarded and the spin columns were re-housed in fresh 2 mL PTs. This step was repeated a further time, with the centrifuge time extended to 3 minutes, at the same speed.

To ensure that all remaining wash buffer had been eliminated from the PAXgene® RNA spin columns the spin columns were placed in fresh PTs and centrifuged for 1 minute at 12,000 x g.

The PT containing the flow-through was discarded and the PAXgene® RNA spin column was placed in a carefully labelled 1.5 mL MCTs for the final elution. A volume of 40 µL of elution buffer (BR5) was pipetted directly onto the PAXgene® RNA spin column (PRC) membrane (in order to achieve maximum elution efficiency). This fusion of the MCT containing the PAXgene® RNA spin column was centrifuged for 1 minute at 12,000 x g (RCF) to elute the RNA. This step was then repeated using the same MCT for each PAXgene® RNA spin column to maximise the total RNA elution.

The eluate was incubated for 5 minutes at 65°C (to denature the RNA) using the shaker–incubator (without shaking). After incubation, the MCTs are placed on ice prior to quality control testing.

2.3.2 Quality Control Analysis of RNA

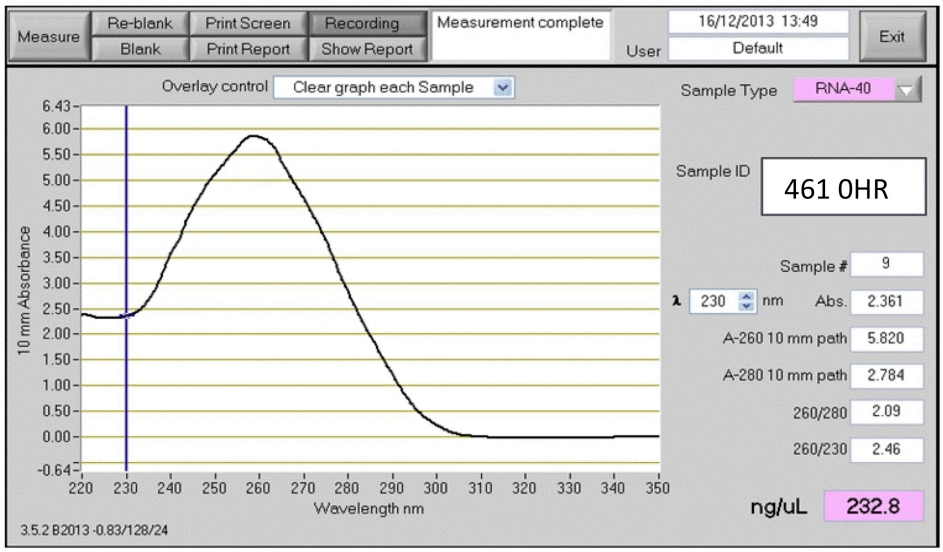
2.3.2.1 NanoDrop™ 2000c

The NanoDrop™ (Thermo Fisher Scientific, USA) uses spectrophotometry, to measure the absorbance of UV visible light transmitted through the sample of interest. It utilises surface tension to hold and measure micro-volumes of samples between two optical pedestals. From this information the machine is able to derive an accurate concentration (in ng/μL) of the sample, as well as specific wavelength absorptions (230, 260 and 280 nm). These values can then be combined as ratios to give an approximation of the purity of the sample isolated.

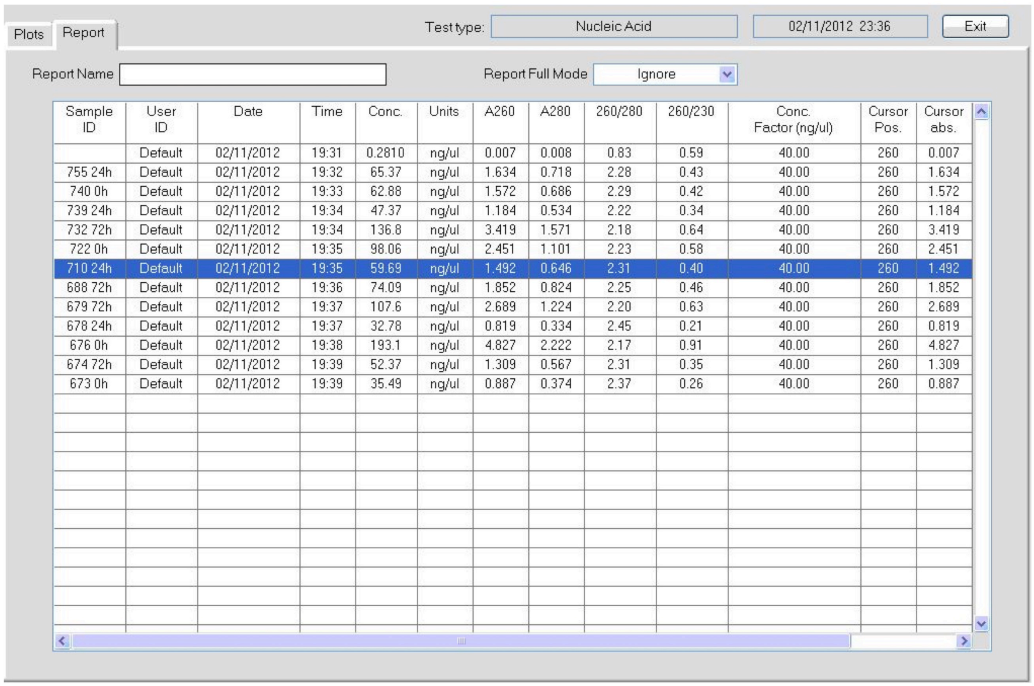
As this machine is commonly used for the analysis of DNA as well as RNA, to prevent contamination from previous samples it was carefully cleaned prior to use with an alcohol swab. Prior to any analysis the machine requires that it be calibrated. Routinely the machine was 'blanked' by pipetting 1 μL of nucleotide free water (NFW) onto the optical pedestal. These pedestals were then wiped with tissue paper and a further 1 μL of NFW was added onto the optical pedestal. The machine then was then run as if to analyse this as an RNA sample, if the resulting concentration was not 0 ng/μL, with a straight-line graphic displayed on the monitor, then the machine was re-blanked.

Following this, the recently isolated RNA samples, stored on ice, were run on the NanoDrop™ with the concentrations, 230, 260 and 280 ng/μL (Figure 2.1) recorded on Microsoft Excel spread sheet (Microsoft Inc., USA). The samples were immediately stored in the -80°C freezer until further analysis.

A



B



Sample ID	User ID	Date	Time	Conc.	Units	A260	A280	260/280	260/230	Conc. Factor (ng/ul)	Cursor Pos.	Cursor abs.
	Default	02/11/2012	19:31	0.2810	ng/ul	0.007	0.008	0.83	0.59	40.00	260	0.007
755 24h	Default	02/11/2012	19:32	65.37	ng/ul	1.634	0.718	2.28	0.43	40.00	260	1.634
740 0h	Default	02/11/2012	19:33	62.88	ng/ul	1.572	0.686	2.29	0.42	40.00	260	1.572
739 24h	Default	02/11/2012	19:34	47.37	ng/ul	1.184	0.534	2.22	0.34	40.00	260	1.184
732 72h	Default	02/11/2012	19:34	136.8	ng/ul	3.419	1.571	2.18	0.64	40.00	260	3.419
722 0h	Default	02/11/2012	19:35	98.06	ng/ul	2.451	1.101	2.23	0.58	40.00	260	2.451
710 24h	Default	02/11/2012	19:35	59.69	ng/ul	1.492	0.646	2.31	0.40	40.00	260	1.492
688 72h	Default	02/11/2012	19:36	74.09	ng/ul	1.852	0.824	2.25	0.46	40.00	260	1.852
679 72h	Default	02/11/2012	19:37	107.6	ng/ul	2.689	1.224	2.20	0.63	40.00	260	2.689
678 24h	Default	02/11/2012	19:37	32.78	ng/ul	0.819	0.334	2.45	0.21	40.00	260	0.819
676 0h	Default	02/11/2012	19:38	193.1	ng/ul	4.827	2.222	2.17	0.91	40.00	260	4.827
674 72h	Default	02/11/2012	19:39	52.37	ng/ul	1.309	0.567	2.31	0.35	40.00	260	1.309
673 0h	Default	02/11/2012	19:39	35.49	ng/ul	0.887	0.374	2.37	0.26	40.00	260	0.887

Figure 2.1 - Nanodrop plot & Data

Nanodrop plot (A), 260/280 ratio & concentrations obtained from total RNA isolated from the PAXgene® system (B).

2.3.2.2 Agilent's 2100 Bioanalyzer

Further quality control was carried out. Although the NanoDropTM gives an accurate yield of RNA that has been isolated, using the 260/280 and 230/260 ratios give more of an approximation of the purity of the sample. As a result a more robust measurement of RNA quality is required.

The bioanalyzer generates an RNA integrity number (RIN) (Schroeder *et al.*, 2006), a value out of 10, which is used to score the quality of isolated samples. A threshold value of 7 has been advocated by some labs to prevent inaccuracies being generated during the qPCR phase.

The system uses a 16 well chip with micro-channels engineered into the glass which then connects the differing wells. During the chip preparation these micro-channels are filled with a polymer and fluorescent dye allowing a circuit to be formed. The chip reader uses 16-pin electrodes, which allow the differing electrodes to read specific wells. As RNA are charged molecules, they are electrophoretically driven by this voltage gradient. In a manner similar to gel electrophoresis, in the presence of a sieving polymer matrix, the molecules are separated by size with smaller fragments moving the furthest. Due to the fluorescent dye mixed with the polymer these RNA strands are detected by laser. This then generates gel-like images (bands) as well as electropherograms (peaks).

Prior to starting this protocol, batches of total RNA samples were removed from the -80°C freezer and allowed to thaw gently on ice. The ladder, provided with the kit was heat denatured at 70°C for 2 minutes and there after stored on ice. The Nano kit (Agilent Technologies, USA) was removed from the refrigerator and allowed to acclimatise at room temperature for 30 minutes. Following this the gel mixture was filtered. 550 µL was aliquoted into a spin filter and centrifuged at 1,500 x g (1-15 Mini-centrifuge, Sigma, UK). The filter was removed from the top of the MCT. Following this the filtered gel could be stored at 4°C for 4 weeks until further use.

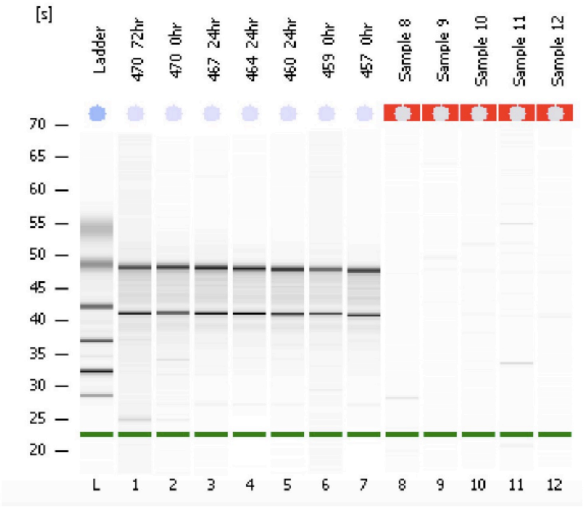
65 µL of filtered gel was aliquoted into a fresh MCT along with 1 µL of pre-vortexed RNA dye concentrate. The MCT was agitated on the vortex (MS3 Chip vortexer, IKA, UK) to ensure complete mixing and then centrifuged 13,000 x g (1-15 Mini-centrifuge, Sigma, UK) for 10 minutes at room temperature. This gel-dye mix was used within 12 hours of preparation.

To load the RNA chip a new chip was placed on the chip priming station and 9 µL of the freshly mixed gel-dye mix was pipetted into the wells marked G. The syringe attached to the chip priming station was positioned at 1 mL and the chip priming station was then closed. The plunger on the syringe was then pushed down until a click was heard. The plunger was then held in this position for exactly 30 seconds and then the clip was released, allowing a minimum of 5 seconds before the plunger was then returned to the 1 mL position.

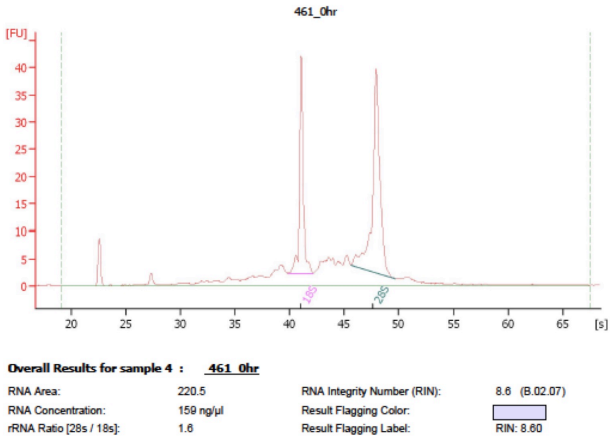
Following this 9 µL of the freshly centrifuged gel-dye mix was also added to the wells marked G. 5 µL of the RNA marker was then added to all 12 of the sample wells in addition to the ladder well. After this was completed 1 µL of sample was added to each of the sample wells and 1 µL of the denatured ladder was finally added to the ladder well. The chip was then agitated on a specifically designed vortexer (Agilent Technologies, USA) for 1 minute at 2400 rpm and then run on the bioanalyzer immediately following this. Prior to running, the electrodes on the bioanalyzer were carefully cleaned with a chip filled with RNAsezap® (Life Technologies, USA) to remove residual RNA which could contaminate the chip, and then washed with a chip containing NFW.

The bioanalyzer system takes approximately 30 minutes to analyse the chip, giving both gel-like images (bands) as well as electropherograms (peaks) and the RIN figure for each sample (Figure 2.2A-C)).

A



B



Overall Results for sample 4 : 461_0hr

RNA Area:	220.5	RNA Integrity Number (RIN):	8.6 (B.02.07)
RNA Concentration:	159 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	1.6	Result Flagging Label:	RIN: 8.60

C

Sample Name	Sample Comment	Status	Result Label	Result Color
470_72hr		✔	RIN: 8.70	
470_0hr		✔	RIN: 7.80	
467_24hr		✔	RIN: 8.60	
464_24hr		✔	RIN: 8.60	
460_24hr		✔	RIN: 8.60	
459_0hr		✔	RIN: 8.30	
457_0hr		✔	RIN: 8.20	
Sample 8		✔	RIN N/A	
Sample 9		✔	RIN N/A	
Sample 10		✔	RIN N/A	
Sample 11		✔	RIN N/A	
Sample 12		✔	RIN N/A	

Figure 2.2 - The Bioanalyzer system

The Bioanalyzer system demonstrating gel-like images (bands), (A) as well as electropherograms (peaks), (B). The bands (A) are sharp and clear with the 28s band displaying approximately twice the intensity of the 18s band indicating that the RNA has not been degraded. The electropherograms (B) display high quality total RNA with the 18s and 28s fragments clearly visible at 40 seconds and 47 seconds. The RIN value for each sample assayed (C).

2.3.3 Reverse Transcription Of RNA To Complementary DNA (cDNA)

In order for the RNA to be analysed by Taqman Qualitative Real-Time Polymerase Chain Reaction it needs to be reverse transcribed to complementary DNA. For all experiments SuperScript® VILO™ (Life Technologies, USA) was chosen based on the efficacy and reproducibility of this product as described in the literature.

In order to standardise the concentration of the mRNA that was isolated the NanoDrop ™ concentration was used to identify the volume of mRNA required to give a total of 300ng of mRNA. If this volume was less than 16 µL then the remainder was made up with NFW in a 96 well plate (Starlab, UK). This gave a total volume of 16 µL at a concentration of 18.75 ng/µL. The SuperScript® VILO™ master mix was removed from the -20°C freezer and rested on ice. A volume of 4 µL of the SuperScript® VILO™ (Life Technologies, USA) master mix was then aliquoted to each of the wells. The plates were then firmly sealed with an adhesive plate seal (Life Technologies, USA), briefly vortexed and then centrifuged (4K15 Bench-top Centrifuge, Sigma, UK) for 10 seconds to ensure adequate mixing of the standardised dilution of the mRNA and the master mix. For each of the plates in addition to the patient samples a positive (lab mRNA sample with a RIN ≥9.5) and a negative control (NFW) was added.

Each of the plates was incubated in the MJ Tetrad 2 DNA Engine cycler (Bio-Rad, UK) using a standardised automated programme of a 25°C incubation for 25 minutes, followed by a 42°C incubation for 60 minutes, with the reaction terminated by incubation at 85°C for 5 minutes. Following the completion of this protocol the cDNA plates were then rested on ice and the contents of each well was carefully transferred into a new 96 (deep) well plate (Starlab, UK) and made up to a working volume of 300 µL with the addition of 280 µL of NFW added to each well to give a final concentration of cDNA of 1 ng/µL. The deep well plates were stored at -20°C until Taqman analysis was performed.

2.3.4 Standardisation Of Taqman Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Quantitative real-time polymerase chain reaction (qRT-PCR) is of great importance in biological research, providing accurate and sensitive expression profiling of candidate genes, offering insight into complex molecular pathways (Zamorano *et al.*, 1996). For this research technique to be reproducible a standardisable technique for calculating gene expression is required (Freeman *et al.*, 1999). Currently there are two main methods in the literature that are used to analyse the raw data from qRT-RCR; absolute and relative quantification (Livak & Schmittgen, 2001).

Absolute quantification describes the input copy numbers of the gene of interest. This is calculated by relating the PCR signal to a standard curve. This is performed when it is necessary to describe the absolute transcript copy number. It allows one to describe the effect of an intervention leading to (e.g.) an increase in the expression of a candidate gene from (e.g.) 500 copies per cell to 1500 copies (Livak & Schmittgen, 2001). Due to these experiments not requiring absolute copy numbers, relative quantification was used.

Relative quantification describes a complex relationship, requiring equations and assumptions. It relates the change in expression of the candidate gene in relation to one or more reference genes. In the qRT-PCR experiments performed as part of this thesis, this methodology was used, utilising the $2^{-\Delta\Delta Ct}$ equation. This relies on a number of factors for this equation to be applied correctly (Livak & Schmittgen, 2001).

The first of these is the assumption that the amplification efficiencies for the candidate and references genes are approximately equal. This is detected by plotting a graph of a series of cDNA dilutions (plotted on a logarithmic scale) against the ΔC_t obtained for all of the candidate genes and reference (housekeeping) genes. This then allows one to calculate the absolute value of the slope of your graph for the target genes. If this slope is close to zero

then the $\Delta\Delta C_t$ methodology may be used, as this signifies that the efficiencies of the candidate and reference genes are similar (Livak & Schmittgen, 2001; Winer *et al.*, 1999).

The Second key factor is the selection of appropriate reference (housekeeping) genes for the experiment. Commonly these are drawn from ubiquitously expressed genes such as GAPDH, ATP5B or EIFA42 as well as many others. In the case of the trauma experiments, mRNA from patients with diagnosed ICU infections, no ICU infections and normal healthy controls were selected in order to provide an accurate representation of the expression levels of all the samples obtained to select the most stable combination of the six reference genes that were analysed in Figure 2.3. The average expression stability (M) is defined as the average pairwise variation between a candidate gene and all other control genes (Vandesompele *et al.*, 2002). The values of the reference genes are then calculated using a VBA applet geNorm (geNorm, 2002; Vandesompele *et al.*, 2002), illustrated in Figure 2.4. As a result ATP synthase subunit beta (ATP5B) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were selected in combination for the qRT-PCR experiments. This was assessed in a similar manner for the perioperative experiments with β2 microglobulin (B2M) and ubiquitin C (UBC) selected.

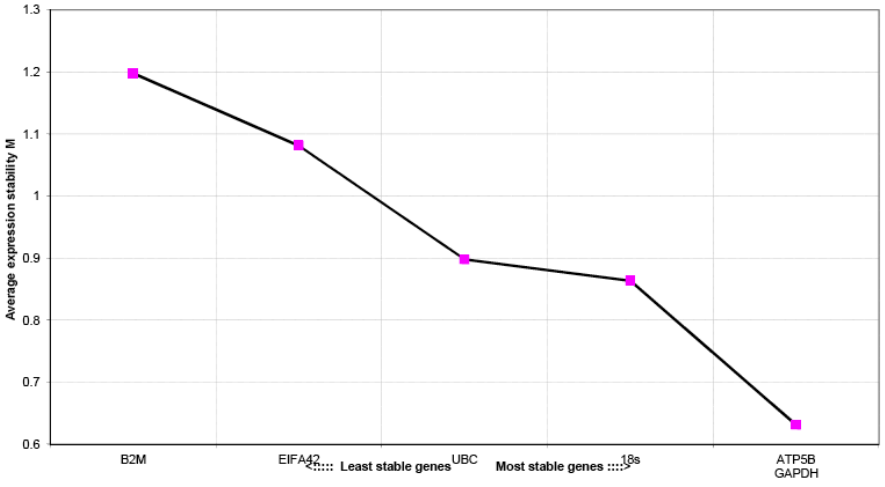


Figure 2.3 - Average expression stability values of the housekeeping genes assessed

A graph illustrating the average expression stability (M) values of the six reference genes evaluated**.**

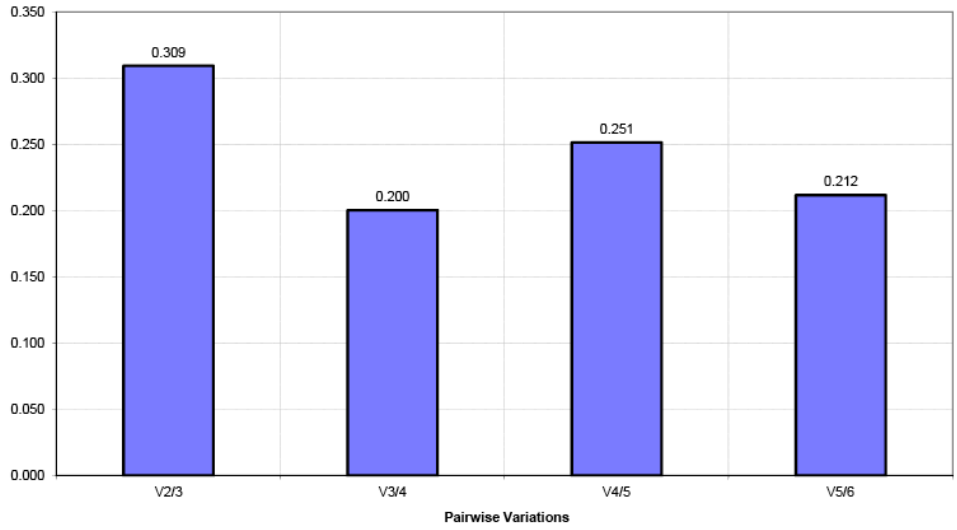


Figure 2.4 - Determining the optimal number of control genes for normalisation

This graph illustrates the optimal number of reference genes that are required for the series of experiments conducted.

Figure 2.4 goes onto illustrate that there is no significant change in the normalisation factor with the addition of further reference genes to the experiment. This confirmed that the ATP5B and GAPDH were adequate as reference genes. This is a trade off between practical considerations (using multiple reference genes) and the accuracy of the data (Vandesompele *et al.*, 2002).

The third assumption is that the amount of starting material for each of the samples is equal and the quality of the mRNA extracted is adequate (Huggett *et al.*, 2005). Concentration was measured using a spectrophotometer (NanoDrop 2000c, as previously described), giving a concentration value (in ng/μL). The quality of the mRNA extracted was qualified via a nano chip on the bioanalyzer 2100, giving an RNA integrity number (RIN), as previously described. This then allowed the creation of a standardised concentration (1 ng/μL) of the mRNA for reverse transcription to an equivalent cDNA concentration (using SuperScript® VILO™ Master mx). As previously described, known positive controls, mRNA with a lab validated expression level and negative controls (NFW), were also included on each plate to minimise errors.

Other precautions were also taken; mRNA during the extraction process was treated with a DNase to prevent non-specific binding of contaminant DNA during the qRT-PCR process. Off the shelf assays were chosen that had been previously validated for accurate binding by publications in peer-reviewed journals. RORγT was the exception to this. This was a custom assay, chosen to span the final two exons of the most common isoforms of this gene, as an off the shelf assay was not available. To minimise pipetting error, robot programmed plate preparation was carried out; sample input was cross-checked prior to the plate being run. Samples were run in triplicate with all assayed primers and probes for that patient sample on the same plate. The mean threshold cycle (C_t) of the three values was used. C_t values that were more than 2 standard deviations from the other 2 samples were re-done.

The C_t is the point when the fluorescence signal is statistically significant to the baseline (Gibson *et al.*, 1996). It is determined from a log-linear plot of the PCR signal versus the cycle umber (Livak & Schmittgen, 2001) and is utilised to provide reproducible quantification. Fluorescence values are recorded during every cycle, representing the amount of the candidate gene amplified at that time point (Higuchi *et al.*, 1993). The more of the template that is present in the well at the start of the reaction means that fewer cycles are required for the C_t to be reached, always in the exponential phase of amplification. This means that the reaction is not limited by reaction components, removing a potential bias source (Bustin, 2000). Too great a primer concentration is another potential source of bias, as this may encourage mispriming and therefore the collection of a non-specific product. With the C_t methodology, this is again minimised (Bustin, 2000).

2.3.5 Taqman Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Polymerase chain reaction (PCR) has revolutionised biology. A further development of this, Taqman chemistry, was first published in 1991 (Holland *et al.*, 1991). It uses a fluorophore covalently attached to the 5'-end of the oligonucleotide probe and a quencher at the 3'-end. Ordinarily the quencher molecule (being in close proximity to the fluorophore)

counteracts the fluorescence emitted by the fluorophore when excited by the cycler's light source via Fluorescence Resonance Energy Transfer (FRET). Probes are designed such that they anneal within a DNA region amplified by a specific set of primers. As the Taq polymerase extends the primer and synthesises the strand, the exonuclease activity of the Taq polymerase degrades the probe that has annealed to the template. Degradation of the probe causes release of the fluorophore, moving it away from the proximity of the quencher, thus allowing its detection. As a result, fluorescence detected is directly proportional to the fluorophore released and therefore the amount of DNA template present in the PCR.

The ABI Prism 7900HT (Life Technologies, USA) contains a built-in thermal cycler with 384-well positions, and is able to detect fluorescence between 500nm and 660nm. Fluorescence is induced during the RT-PCR by distributing laser light to all 384 samples contained in thin-walled reaction tubes via a multiplexed array of optical fibres. The resulting fluorescent emission returns via the same fibres.

The ABI prism establishes the levels of background fluorescence for each particular run. An algorithm is used to define a fluorescence background threshold. The algorithm then searches the data from each sample for a point that exceeds the baseline. The cycle at which this occurs is defined as the Crossing Threshold (C_t). After analysis by the SDS software (Applied Biosystems, USA), the resulting C_ts are used to establish quantitative relationships with the assayed cDNA. The fewer cycles it takes to reach a detectable level of fluorescence, the greater the initial copy number of the target nucleic acids.

Methods to standardise this process, thus reducing the likelihood of errors being generated, have previously been outlined in this thesis. As part of the standard running protocol for the ABI Prism cycling conditions of 50°C for 2 minutes followed by 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute were used. The raw Ct values were exported via SDS to a Microsoft Excel spread sheet (Microsoft Inc., USA) where

they were analysed using the delta-delta methodology of relative quantification (as previously described).

Standard curves for each of the assays used were performed with six differing dilutions (0.25 – 15 ng/μL) of cDNA. This showed correlation coefficients >0.99, demonstrating a precise log-linear relationship.

2.3.6 Enzyme Linked Immuno-Sorbent Assay (ELISA)

ELISA is another well-established technique, which is commonly used to quantify the concentration of a chosen protein in a liquid sample. The technique ultilises antigen-antibody affiliations, resulting in the creation of an antibody-antigen 'sandwich'. The final layer of the sandwich is a flurophore whose emissions are then detected by a plate reader (at 450nm). The density of the signal mirrors the presence of the target substance, enabling the concentration of the substance to be calculated. For the purposes of this thesis it was elected to analyse the concentration so the two most reliably detected cytokines IL-6 and IL-10 were used (Baigrie *et al.*, 1992; Fragkou *et al.*, 2014; Giannoudis *et al.*, 2000; Mokart *et al.*, 2005b). Our group had previously tried the LUMINEX platform for multiplex cytokine analysis but had disappointing results with key cytokines such as TNF-α and IFN-γ being variably detectable.

For analysis citrated plasma was used; this was collected as previously described. The 2.0 mL Corning® cryogenic vials (Corning Incorporated, NY, USA) were allowed to acclimatise at room temperature for a period of 4 hours. Following acclimatisation they were centrifuged at 3000 RPM for 10 minutes in order to pellet any debris that may have been present in the sample. For both the IL-6 and IL-10 kits it was recommended to use a 1 in 2 dilution with the provided Standard Diluent Buffer. Each sample was gently mixed by pipette.

A wash buffer solution, supplied as 25x concentrated was diluted with the recommended volume of deionised water. This was added to the EL_x50 Auto strip washer (BIO-TEK Instruments, INC, Vermont, Canada) and the machine was primed prior to use. It was programmed to aspirate from the wells, and then fill the wells with 0.4 mL of diluted wash solution. This was allowed to soak for 15 seconds, and then aspirated. These steps were programmed to occur four times for each washing step.

The standards were provided in freeze-dried form and were reconstituted in their glass vials with the standard diluent buffer supplied. Following reconstitution the vials were gently mixed by flicking and a serial dilution was performed giving concentrations of 500, 250, 125, 62.5, 31.2, 15.6, and 7.8 pg/mL for the IL-6 ELISA plates and 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 pg/mL for the IL-10 ELISA plates. 100 µL of the standards were plated sequentially in duplicate from wells A1 and B1 to A7 and B7 of the pre-coated 96 well ELISA plate. The final wells (A8 and B8) were plated with Standard Diluent Buffer (B) to act as the negative control (Figure 2.5).

Patient samples were diluted 2:1 and were plated along with the standards in duplicate on each plate. For the IL-10 assay samples were allowed to incubate for two hours in a SW-20C Water Bath (Julabo, Germany). For the IL-6 assay in addition to the patient samples and standards, a 50 µL volume of biotinylated anti-IL-6 (Biotin Conjugate) solution was added to each well aside from the chromogen blanks (A8 and B8). This was incubated at room temperature for two hours.

Following this step, both plates were washed on the EL_x50 Auto strip washer (BIO-TEK Instruments, Canada) plate washer, using the protocol previously described. Using a multichannel pipette a 100 µL volume of biotinylated anti-IL-10 (Biotin Conjugate) solution was added to each of the wells of the IL-10 plate except the chromogen blanks (A8 and B8). This was incubated at room temperature for one hour. The IL-10 plate was washed as previously described.

Using a multichannel pipette, a 100 µL volume of Streptavidin-HRP Working Solution was added to each well of both plates except the chromogen blanks. This working solution had been made up using 120 µL of the concentrated solution supplied with the kit and 12 mL of Streptavidin-HRP Diluent. The plates were incubated at room temperature for 30 minutes and re-washed as described.

Again using a multichannel pipette, a 100 µL volume of Stabilised Chromogen was added to each well of both plates. Addition of this solution caused the contents of the wells to change colour to blue. This was incubated in the dark for a period of 25 minutes. Following this a 100 µL volume of Stop Solution was added to each well of both plates. This was mixed by gently tapping the side of each of the plates. The addition of this solution caused a colour change from blue to yellow. The plates were protected from light until they were read on a Synergy HT Plate Reader (Bio-Tek, Canada) at 450nm within two hours of the stop solution having been added.

The data for each plate was exported into a Microsoft Excel spreadsheet (Microsoft Inc., USA), from where it was analysed. Optical densities for each standard were plotted using Graph Pad Prism version 6.0g (GraphPad Software Inc, USA). These data were log transformed to create a standard curve. Unknown plasma concentrations were obtained from the standard curve and transformed back into raw figures. Samples lying outside the limits of detection for the standard curves were re-plated at an altered concentration based on whether they were below or above the standard curve values. The duplicate values were examined and if a difference between the two values obtained varied greater than 15% then these samples were re-plated. A mean value of the two concentrations was used for analysis.

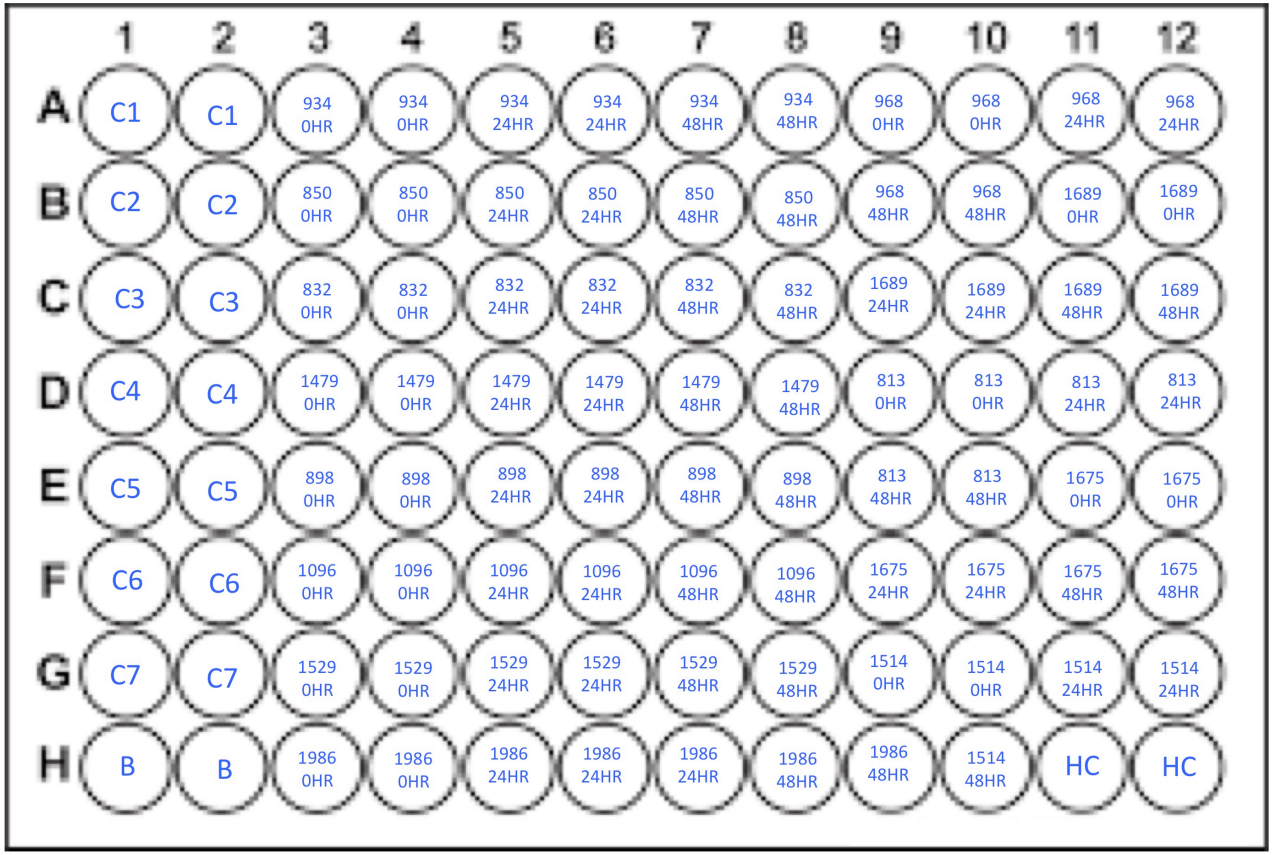


Figure 2.5 - ELISA plate outline

2.3.7 Flow Cytometry

The use of flow cytometry in a clinical capacity is increasing; this has been largely driven by oncology with complex immune-phenotyping taking place to guide treatment options. However more recently this has been introduced into critical care research (Monneret & Venet, 2015). The current gold standard for the identification of an immunocompromised patient on the critical care unit is by determining the number of HLA-DR molecules per monocyte (Ab / C ($CD14^+$)), (Docke *et al.*, 2005). This test has previously been used in order successfully to randomise patients to receive immune stimulants (Meisel *et al.*, 2009). As a result this test was employed as a standardised measure of the immune-competence of these two patient cohorts.

Blood is collected in a 4 mL EDTA anticoagulated tube with all samples stained <one hour following collection. The EDTA tube was gently agitated for 2 seconds on the vortex (Heidolph Reax top test tube shaker, Germany). 50 µL of blood was then aliquoted from the collection tube and added to a 5 mL FACS tube (Becton Dickinson, UK). The Quantibrite® Monocyte/HLA-DR antibody (Becton Dickinson, UK) was retrieved from the fridge and, after being gently mixed with a pipette, 20 µL of this was added to the FACS tube using a fresh pipette tip. This process was carried out in duplicate. The FACS tubes were gently agitated for 5 seconds on the vortex and then incubated in the dark for 30 minutes at room temperature. A previously prepared lysis and fixation solution (FACS lysis solution Becton Dickinson, UK) diluted 1:10 with deionised water (Sigma, UK)) was retrieved from the refrigerator. This was warmed in the water bath at 37^{o}C (SW-20C Water Bath, Julabo, Germany).

Following 30 minutes of incubation with the antibody the lysis (containing fixative) buffer was removed from the water bath and 500 µL was aliquoted into each of the FACS falcon® tubes (Becton Dickinson, UK). These were gently mixed by vortexing and re-incubated in the dark for 15 minutes. During this time frame the solution changes from opaque to translucent.

After 15 minutes incubation, 1 mL of FACS buffer (2% fetal bovine serum (FBS) and Phosphate Buffered Saline (PBS) (StemCell, France)) is added to each tube. Caps were then added and the tubes were centrifuged at 300 x g (RCF) for 5 minutes with the brake on (3SR+ Bench-top Centrifuge, Thermo Scientific, UK). Following this the supernatant was aspirated with a pipette and the pellet was resuspended (by vortexing) in 500 µL of FACS buffer. The tubes were then immediately run on an LSR II flow cytometer (Becton Dickinson, UK) and 1,500 monocyte events were collected for each tube, gating on $CD14^+$ cells and then measuring the HLA-DR geometric mean fluorescent intensity (gMFI) of those $CD14^+$ cells.

In addition to this as part of the protocol Quantibrite® Phosphatidylethanolamine (PE) coated beads (Becton Dickinson, UK) were run each week. These allowed gating (on the histogram setting) of HLA-DR expression, dividing them into low, low-medium, medium-high and high expression levels. These individual gMFI values for each of the four-gated parameters were calculated. A line of linear regression of Log_{10} PE molecules per bead against Log_{10} fluorescence is plotted using the following equation:

$$y = mx + c$$

Where y is the log_{10} FL2 fluorescence and x is log10 PE molecules / bead population.

In order to determine bound anti-HLA-DR antibody (AB / c) per monocyte, the log_{10} FL2 geometric means are substituted with the log_{10} FL2 median value of the monocyte population, and the equation is solved for x = log_{10} Ab / C. The antilog is determined to obtain the Ab / C (Docke *et al.*, 2005). The gating strategy is outlined in Figure 2.6 on the next page.

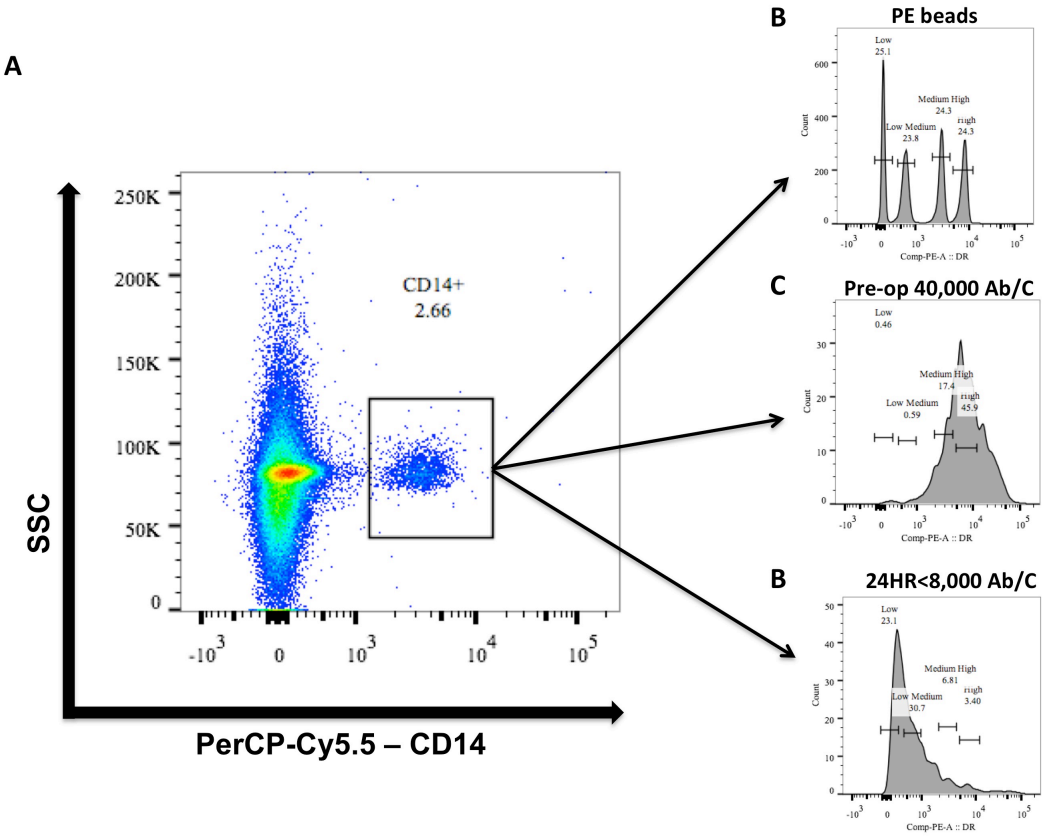


Figure 2.6 - Representitive flow cytometry plot for gating lysed whole blood samples

Cells were gated on CD14 (A). PE coated beads were used to calibrate the experiment, gave four defined population; low, low-medium, medium-high and high to allow accurate gate placement (B). A histogram plot showing preoperative (C) and postoperative (D) HLA-DR expression on the gated $CD14^+$ cells. Using g(MFI), linear regression was performed to calculate the mAb / C.

Following cell culture PBMCs were stained in a similar manner, using 10 µL of Quantibrite® Monocyte/HLA-DR antibody (Becton Dickinson, UK) per 300,000 PBMCs in a 5mL falcon® tube (Becton Dickinson, UK). These were incubated at room temperature for 30 minutes, washed and resuspended as previously described. 2,000 monocyte events were collected for each tube, gating on forward scatter and side scatter, then $CD14^+$ cells. Following this the positive cells were then assessed for their HLA-DR expression using geometric MFI. The gating strategy is outlined in Figure 2.7.

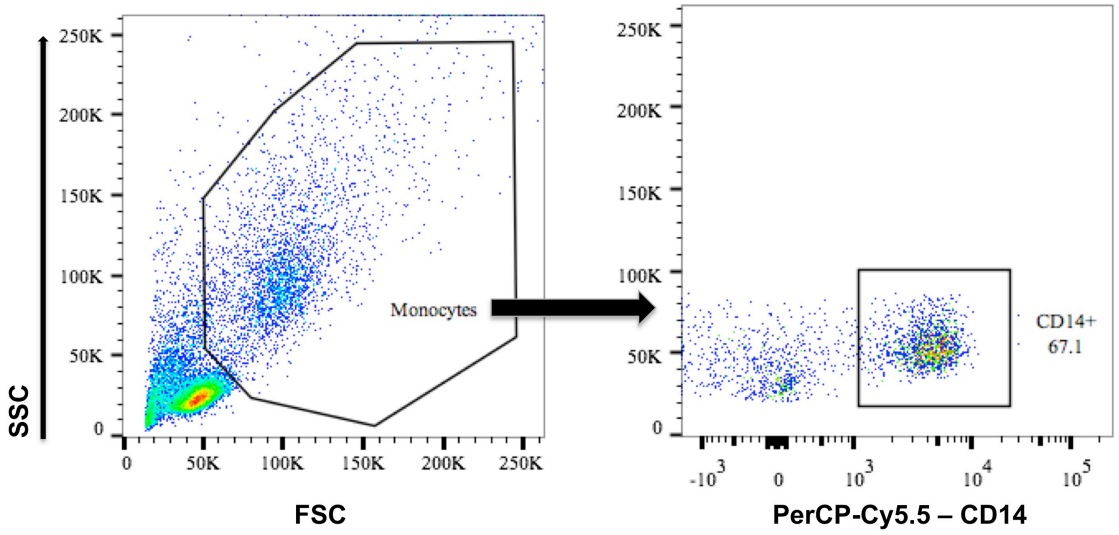


Figure 2.7 - Representative flow cytometry plot for gating cultured PBMCs

Cells were gated according to their side and forward scatter properties in order to identify the monocyte subpopulation. This subpopulation was then gated according to CD14 expression. $CD14^+$ cells were then analysed for HLA-DR geometric mean intensity (*g*MFI).

2.3.8 Cell Culture

8 mL of peripheral blood were collected in BD Vacutainer™ Sodium Citrate CPT™ tubes (Becton Dickinson, UK) from a healthy control cohort. These were immediately centrifuged at 1,800 RCF for 30 minutes at 20°C with the brake off. The peripheral blood mononuclear cell (PBMC) layer was isolated and washed in Phosphate Buffered saline (Life Technologies, Carlsbad, CA) containing 2% human albumin solution (Sigma, UK). Cells were then counted with a haemocytometer under a light microscope.

Pooled healthy PBMCs were aliquoted ($3x10^5$ cells per well) into a 96 well plate and cultured in duplicate with Gibco® RPMI 1640 medium (Life Technologies, Carlsbad, CA) containing 30% patient serum, which was taken either preoperatively (preop) or at 24 hours postoperatively, for 20 hours at 37°C with 5% CO_2 (CB 150, Binder).

2.3.8.1 Neutralising Experiments

Pooled healthy PBMCs were pre-incubated with an Fc Block™ (Becton Dickinson, UK) for 30min then incubated with 30% 24 hour serum and media pre-incubated with either IL-6 neutralising antibody 15 ng/mL (R&D systems, UK) or 5-10 μg/mL of IL-10 neutralising antibody (R&D systems, UK) for a minimum of 1 hour or with control non-specific goat IgG at the same dose (IL-6, IL-10: R&D systems, UK). These were cultured as described above.

2.3.8.2 Stimulating Experiments

Pooled healthy PBMCs were incubated with 30% serum from either baseline or 24 hours with and without 250 International Units of IFN-γ (R&D systems, UK) or Granulocyte-macrophage colony-stimulating factor (GM-CSF) at a range of doses (2 – 20 ng/mL) (R&D systems, UK). These were cultured as described above.

Chapter Three: The Influences Of Polytrauma & Allogeneic Transfusion On T-Helper Cell Related Candidate Gene Expression

This Chapter contains data from the following published works:

'Association between gene expression biomarkers of immunosuppression and blood transfusion in severely injured polytrauma patients' **Torrance HD**, Brohi K, Pearse RM, Mein CA, Wozniak E, Prowle JR, Hinds CJ, O'Dwyer MJ. *Annals of Surgery*. 2015; 261:751-9. (ePub Mar 2014) PMID: 24670848

'Changes in gene expression following trauma are related to the age of transfused packed red blood cells' **Torrance HD**, Vivian ME, Brohi K, Prowle JR, Pearse RM, Owen HC, Hinds CJ, O'Dwyer MJ. *Journal of Trauma and Acute Care Surgery*. 2015; 78:535-42. PMID: 25710424

'Epigenetic regulatory pathways involving micro-RNAs may modulate the host immune response following major trauma' Owen HC, **Torrance HD**, Jones TF, Pearse RM, Hinds CJ, Brohi K, O'Dwyer MJ. *Journal of Trauma and Acute Care Surgery*. 2015; 79:766-72. PMID: 26496100

This paper reported IL-10, IL-12 & TNF-α gene expression data from this Chapter.

3.1 Introduction

Trauma remains one of the most frequent causes of death in those aged under 45 (Polinder *et al.*, 2012). As early mortality following trauma falls, attributable in part to advances such as the widespread introduction of damage control surgery and improved pre-hospital care (Harris *et al.*, 2012), late onset sepsis and multi-organ failure have now become the most frequent cause of complications and subsequent death in these patients (Lord *et al.*, 2014). An enhanced susceptibility to acquiring infection has been noted, with ventilated trauma patients' risk of pneumonia being four times greater than ventilated non-trauma patients (Magret *et al.*, 2010). The development of sepsis, from any source, in trauma patients is associated with a trebling of the mortality rate (Osborn *et al.*, 2004).

The inflammatory paradigm in relation to severe trauma has changed substantially over time and this is characterised in more detail in Chapter one of this thesis. In short, the original description of an initial systemic inflammatory response syndrome (SIRS) followed by a compensatory anti-inflammatory response syndrome (CARS) (Dewar *et al.*, 2009; Keel & Trentz, 2005; Mannick *et al.*, 2001; Moore *et al.*, 1996) has been supplanted by a description of simultaneous activation of inflammatory and anti-inflammatory pathways (Xiao *et al.*, 2011). More specifically, it is now suggested that trauma induces activation of innate immune pathways, whilst concurrently suppressing adaptive immune pathways (Kasten *et al.*, 2010). Precisely how swiftly the body mounts a response is unclear but recent data suggest the presence of altered inflammatory pathways within 12 hours and possibly within 4 hours of severe trauma (Xiao *et al.*, 2011).

The Immunological picture is however clouded by blood transfusion, as this can be associated with unintended morbidity (Hill *et al.*, 2003). Allogeneic blood products are transfused in up to 9% of trauma patients, with one third of this group requiring massive transfusion (Como *et al.*, 2004). In the US approximately 10% of all red cell transfusions are utilised supporting trauma patients (Dep.Health, 2011). The immunomodulating qualities of

allogeneic blood have long been appreciated and have even been exploited to prevent renal allograft rejection in the era prior to the development of effective immunosuppressants (Opelz & Terasaki, 1978). Clinical consequences of immune modulation by allogeneic blood include an increased susceptibility to infections and cancer recurrence. These transfusion-related immune modulatory (TRIM) effects of allogeneic blood transfusions are particularly well described in the perioperative period (Cata *et al.*, 2013). Although infections have been previously associated with allogeneic blood transfusion in the setting of trauma some of these data are historical and precede the routine use of leukodepleted blood products (Flores *et al.*, 2001; Hill *et al.*, 2003).

In the UK PRBCs can be stored for a maximum of 35 days prior to transfusion and in some countries for up to 42 days. During this time 'storage lesions' may develop which include morphological changes, acidosis, decreased 2,3 diphosphoglycerate and adenosine triphosphate, lipid peroxidation and apoptosis (Bennett-Guerrero *et al.*, 2007). Although the vast majority of currently available evidence is composed primarily of retrospective, non-randomised studies, there is a suggestion that the administration of older PRBCs is more likely to be associated with nosocomial infections and an increased mortality (Lelubre *et al.*, 2009; Zimrin & Hess, 2009). The association between the duration of storage of PRBCs and poor outcome is particularly well described in trauma patients (Keller *et al.*, 2002; Leal-Noval *et al.*, 2008; Murrell *et al.*, 2005; Offner *et al.*, 2002; Weinberg *et al.*, 2008; Zallen *et al.*, 1999) and intensive care unit (ICU) patients (Fernandes *et al.*, 2001; Hebert *et al.*, 2005; Marik & Sibbald, 1993; Purdy *et al.*, 1997; Sakr *et al.*, 2007; Taylor *et al.*, 2006). However, the precise mechanism by which PRBCs stored for prolonged periods increase susceptibility to infections remains elusive.

As a consequence it remains uncertain whether allogeneic blood transfusion as well as the age of the units of PRBCs transfused compounds an inherently immunosuppressive environment such as may be seen following severe trauma.

The development of trauma-induced immunosuppression has been strongly linked with subsequent septic complications (Cheron *et al.*, 2010; Tschoeke & Ertel, 2007). Specifically, decreasing monocyte Human Leukocyte Antigen-DR (mHLA-DR) expression in the early stages following trauma is associated with an increased propensity to subsequently develop infection (Cheron *et al.*, 2010). Although quantification of mHLA-DR expression is gaining widespread acceptance as a biomarker for monitoring immunoparalysis and has been utilised in several interventional clinical trials of immunostimulants (Meisel *et al.*, 2009; Nakos *et al.*, 2002), decreasing mHLA-DR expression does not always equate either to an increased mortality (Perry *et al.*, 2003) or even to an increased incidence of infectious complications (Haveman *et al.*, 2006). These inconsistencies, coupled with the need for a flow cytometer to perform the assay, have limited its more widespread introduction into the clinical setting (Fumeaux & Pugin, 2006).

An alternative strategy to characterise the immune response is to quantify the gene expression of a number of key cytokines and transcription factors which, when analysed together give an indication of the overall systemic inflammatory milieu. Previously this approach has been used to characterise the human immune response to infection (O'Dwyer *et al.*, 2006; O'Dwyer *et al.*, 2008; Pachot *et al.*, 2005) and as a predictor of perioperative infectious complications (White *et al.*, 2011).

This Chapter was designed first to document and describe very early temporal changes in inflammatory pathways in response to severe polytrauma and to describe associations between patterns of gene expression and important clinical outcomes. Second, it was designed to explore the hypothesis that blood transfusion contributes to an immunosuppressed phenotype in severely injured patients and may be associated with an increased risk of infectious complications. And third to assess the influence that older blood has when transfused. The genes selected for this thesis were chosen based on their proven utility in acting as surrogate

measures of the activity of specific immune pathways (O'Dwyer *et al.*, 2008; Pachot *et al.*, 2005).

3.3 Methods

The Methods for this Chapter are fully outlined in Chapter two of this thesis.

3.4 Results

A total of 112 ICU patients suffering severe traumatic injury as their admission diagnosis were enrolled between September 2010 and October 2012.

Table 3.1 shows the baseline characteristics of these patients. The 16 healthy controls had a median age of 28 (IQR 25-29) and consisted of 10 males.

The results of the analysis of 11 cytokines are included. The pathways that these cytokines describe are outlined in Table 3.1.

3.4.1 Immediate Immunological Response To Severe Trauma

All baseline blood samples (Time 0) were taken within 2 hours of the injury. Baseline interleukin 10 (IL-10) mRNA levels were raised in comparison to the control group ($p<0.0001$, Figure 3.1A).

In comparison with the control group Transforming Growth Factor beta (TGFβ), IL-23, RAR-related orphan receptor gamma (RORγt), forkhead box P3 (FOXP3), GATA-3 Tumor necrosis factor alpha (TNF-α), IL-23, IL-27 IL-12p35 and T-bet mRNA, mRNA levels were lower at baseline (Figure 3.1B-J, all $p<0.0001$). Interferon gamma (IFN-γ), levels at baseline were raised significantly compared to those observed in the control group ($p=0.001$, Figure 3.1K).

There was a modest correlation between ISS and IL-10 (Spearman's ρ = 0.28, p=0.004, n=99 (Supplementary Figure 1)) and between ISS and TGFβ (Spearman's ρ = 0.23, p=0.02, n=99) mRNA levels at baseline.

Early blood transfusion, as defined by transfusion prior to the baseline bloods being taken, was not associated with ISS or with subsequent mortality.

3.4.2 Change in cytokine gene expression over time

3.4.2.1 Anti-Inflammatory/T_{reg}/T_h2 Cytokines

IL-10 mRNA levels rose further over the initial 24 hours following trauma (p<0.0001) and then fell from 24 hours to the 72 hour time point (Figure 3.2A) although these levels remained greater than the time 0 IL-10 mRNA levels (p=0.01) for comparison of control versus time 72). GATA-3 mRNA levels fell further over the initial 24 hours (p<0.0001) and again when measured at 72 hours (p=0.03, Figure 3.2B).

TGF-β mRNA levels further decreased over the first 24 hours and then remained unchanged between 24 hours (p<0.0001) and the 72 hour time point (p=0.32, Figure 3.2C).

FOXP3 mRNA levels fell further over the initial 24 hours (p<0.0001) and levels at 72 hours were then unchanged (p=0.11, Figure 3.2D).

3.4.2.2 Pro-Inflammatory/T_h1 Cytokines

TNF-α mRNA levels increased over the initial 24 hours (p<0.0001). No significant change occurred in TNF-α mRNA levels from 24 hours to the 72 hour time point (p=0.10, Figure 3.2E).

Table 3.1 - Selected cytokines & transcription factors & their related pathways

Cytokine	Immune Pathway	Contributes to anti (-) or pro (+) Inflammatory phenotype*
TNF-α	Common end product of many innate & adaptive immune pathways	+
IFN-γ	T_h1 effector cytokine	+
IL-12	Promotes differentiation to T_h1 effector cells	+
T-bet	Transcription factor utilised by T_h1 cells	+
IL-23	Promotes differentiation to T_h17 phenotype	+
IL-27	Inhibits differentiation to T_h17 phenotype	-
RORγT	Transcription factor utilised by T_h17 cells	+
IL-10	Anti-inflammatory cytokine produced by many T cell subtypes & some macrophages	-
FOXP3	Transcription factor utilised by naturally occurring $CD4^+CD25^+$ T_{reg} cells	-
GATA-3	Transcription factor utilised by T_h2 cells	-
TGF-β	Induces widespread apoptosis, promotes T_{reg} & T_h17 cell development	-

*Cytokines and transcription factors will have diverse actions under different conditions and the descriptions above are primarily for illustrative purposes and are not exhaustive.

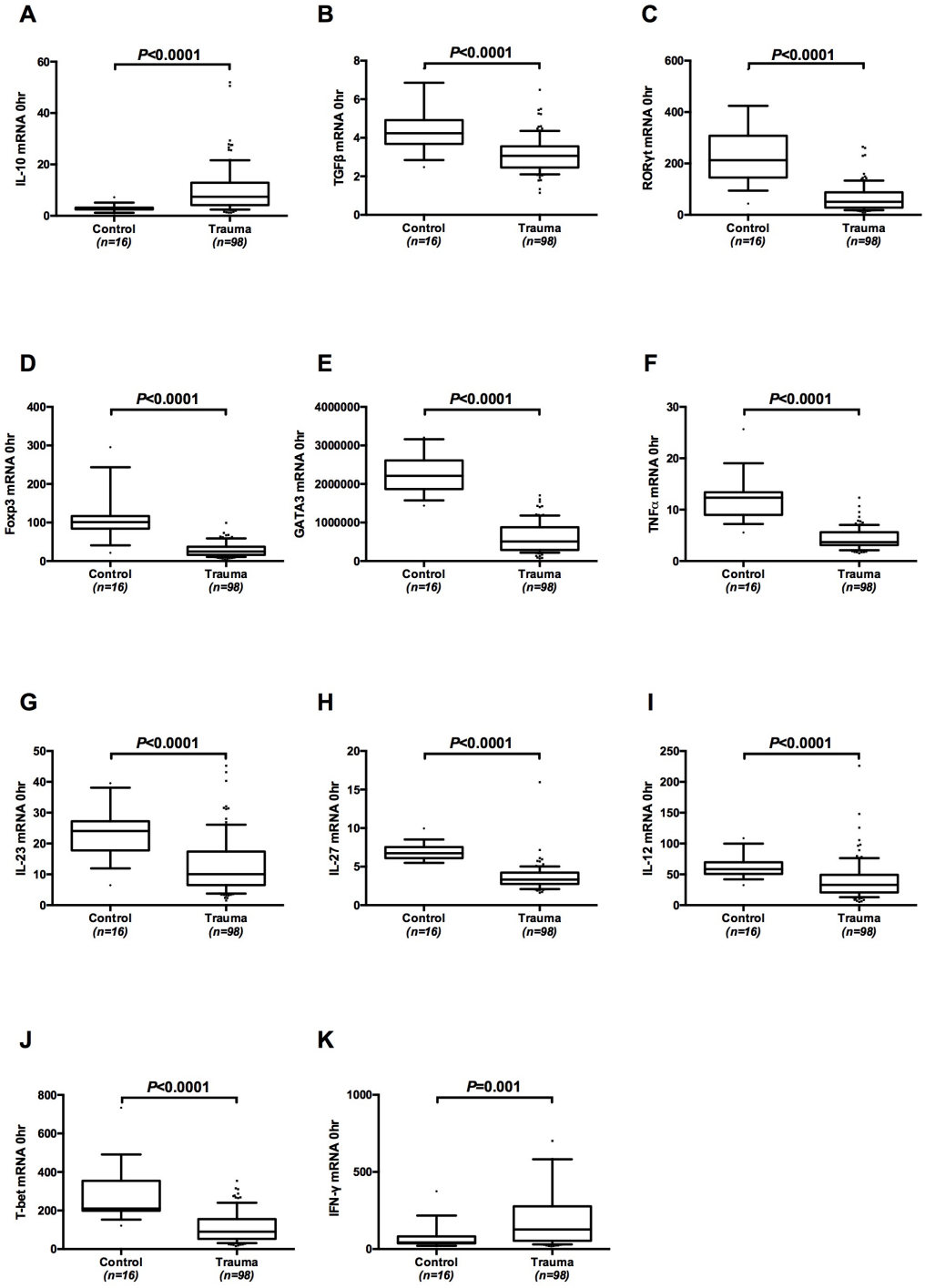


Figure 3.1 - Changes in mRNA between polytrauma patients on admission & healthy controls

Candidate gene mRNA levels assayed from healthy controls (*left*) or at 2 hours following polytrauma (*right*) Graphs A–K, Median, interquartile range (*box*) and the 10th and 90th percentile (*whiskers*). All results are expressed as a relative quantification ratio between candidate and reference genes.

Table 3.2 - Patient characteristics of those suffering polytrauma

	All Patients *(n=112)*	**Infection** *(n=68)*	**Infection Free** *(n=44)*	***p*-value**
Age	40.5 (29.3-57)	39.5 (30-53.3)	41 (28.3-62.3)	*Ns*
Male	88 (79%)	52 (76%)	36 (82%)	*Ns*
ISS	29 (20-36)	30 (21-38)	26 (20-34)	*Ns*
Head Injury	56 (50%)	36 (53%)	20 (45%)	*Ns*
Blunt	101 (90%)	63 (93%)	38 (86%)	*Ns*
Penetrating	11 (10%)	5 (7%)	6 (14%)	*Ns*
Scene sBP	123 (99-138)	123 (96-141)	117 (102-134)	*Ns*
ED sBP	121 (102-139)	120 (93-136)	124 (103-124)	*Ns*
Base Deficit (0hr)	4.1 (1.88-7.01)	4.4 (2.0-9.0)	3.4 (0.1-5.8)	0.03
Lactate (0hr)	3.2 (1.6-5.0)	3.4 (1.9-5.5)	2.6 (1.3-4.3)	*Ns*
PRBCs (P/H)	0 (0-0)	0 (0-1)	0 (0-0)	*Ns*
Crystalloid (P/H)	100 (0-500)	250 (0-500)	0 (0-400)	0.05
HTS (P/H)	0 (0-0)	0 (0-0)	0 (0-0)	*Ns*
PRBCs (24hr)	4 (0-8)	4 (0-8)	3 (0-7.8)	*Ns*
FFP (24hr)	0 (0-6)	0 (0-8)	0.8 (0-6)	*Ns*
Cryo (24hr)	0 (0-2)	0 (0-2)	0 (0-2)	*Ns*
Platelets (24hr)	0 (0-1)	0 (0-1)	0 (0-1)	*Ns*
Crystalloid (24hr)	2750 (1700-4700)	2720 (1672-4786)	2750 (1925-3513)	*Ns*
Colloid (24hr)	1375 (500-2000)	1500 (650-2063)	875 (0-2000)	0.03
HTS (24hr)	0 (0-0)	0 (0-0)	0 (0-0)	*Ns*
MOD	43 (38%)	38 (56%)	5 (11%)	0.001
Survival to Discharge	92 (82%)	60 (88%)	32 (73%)	0.05

Data are expressed as median and interquartile range or absolute counts with percentages in parenthesis. *Colloid*, (mL). *Crystalloid*, (mL). *ED*, Emergency Department. *FFP*, Fresh frozen plasma (Units) *HTS*, hypertonic saline (mL). *ISS*, injury severities score. *Ns,* non-significant. *MOD*, multiple organ dysfunction: This was defined, using the SOFA score, as the presence of ≥2 organs from the SOFA score being ≥3 in 24 hours. *Ns,* non-significant. *P/H*, Pre-hospital. *PRBC*, packed red blood cells (Units).

Table 3.3 - Frequency & location of nosocomial infections following polytrauma

Infection	Frequency
Infection	68 (61%)
Pneumonia	45 (40%)
Wound Infection	23 (21%)
Bacteraemia	15 (13%)
UTI	10 (9%)
Other	7 (6%)
Fungaemia	3 (3%)

Variables are expressed as absolute counts and percentages of the total cohort in parenthesis. Some patients suffered more than one infection. *UTI*, Urinary Tract Infection.

IFN-γ mRNA levels fell over the initial 24 hours to a level that was lower than the control values (p=0.009). There was no change in IFN-γ levels from 24 hours to the 72 hour time point (p=0.20, Figure 3.2F). IL-12p35 mRNA levels fell over the initial 24 hours (p<0.0001), with levels continuing to fall further from 24 hours to the 72 hour time point (p=0.004, Figure 3.2G). T-bet mRNA levels fell over the initial 24 hours (p<0.0001), and continued to fall between the 24 hours and the 72 hour time point (p=0.04, Figure 3.2H).

3.4.2.3 T_h17 Cytokines

IL-23 levels fell further over the initial 24 hours (p<0.0001) and were unchanged from the 24 hour time point to the 72 hour time point (p=0.15, Figure 3.2I). IL-27 mRNA levels were unchanged from baseline to 24 hours (p=0.06) but fell between the 24 to the 72 hour time point (p<0.0001, Figure 3.2J). RORγt mRNA levels decreased further over the initial 24 hours (p<0.0001) and then remained unchanged from 24 hours to 72 hours (p=0.40, Figure 3.2K). There was no correlation between the magnitude of change in cytokine mRNA levels and ISS or between mRNA levels at 24 hours and ISS.

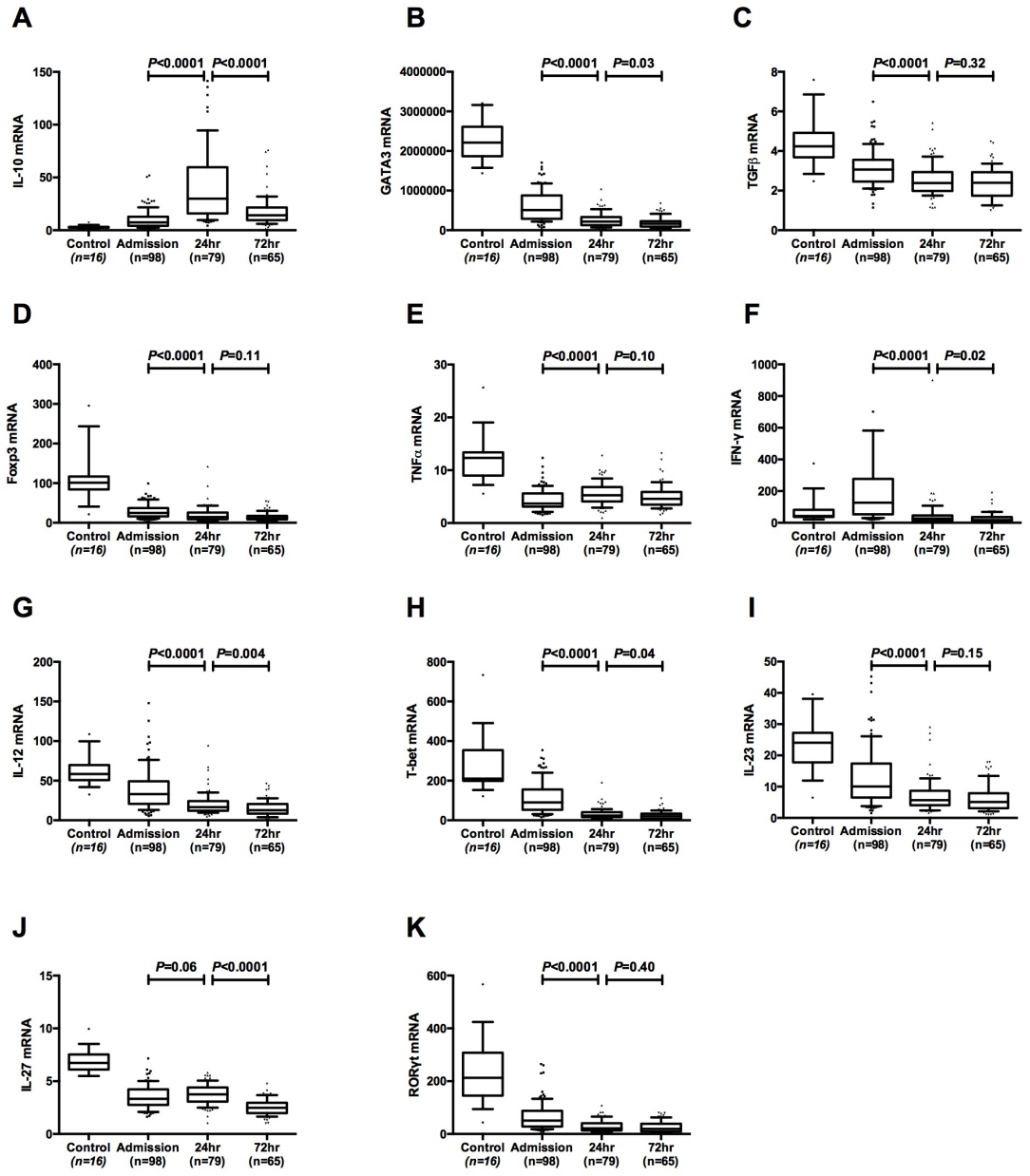


Figure 3.2 - Temporal changes in mRNA expression in polytrauma patients on admission, at 24 & 72 hours

Candidate gene mRNA levels assayed from healthy controls (*Control*), at 2 hours following polytrauma (*Admission*), at 24 hours (*24hr*) and 72 hours (*72hr*) following admission. Graphs A–K, Median, interquartile range (*box*) and the 10th and 90th percentile (*whiskers*). All results are expressed as a relative quantification ratio between candidate and reference genes.

3.4.3 Incidence of infection

Sixty eight (61%) patients developed an infection during their ICU admission. Pneumonia was the most frequent infection (n=45 (40%)) followed by wound infection (n=23 (21%)) (Table 3.3) Following exclusion of likely contaminants bacteraemia was recorded in 15 (13%) patients. The median time to the development of any infection was 4.5 days (2-7). The median time to the development of pneumonia was 4.5 days (3-6.75) and to a bacteraemia was 8 days (5-10.5).

Those patients receiving antibiotics on admission to hospital were more likely subsequently to develop infection. This relationship was independent of ISS, presence of head injury, presence of thoracic injury or the presence of a penetrating injury.

3.4.4 Cytokine gene expression and infection

At both the 24 hour (p=0.005) and the 72 hour (p=0.04) time points greater IL-10 mRNA levels were associated with subsequent infection (Figure 3.3A, B). Higher IL-10 mRNA levels at 24 hours were observed in those patients who subsequently developed a blood stream infection (p=0.0005, Figure 3.3C) and pneumonia (p=0.04, Figure 3.3D). Those patients whose T-bet levels fell the most over the initial 72 hours were more likely to develop infection (p=0.004).

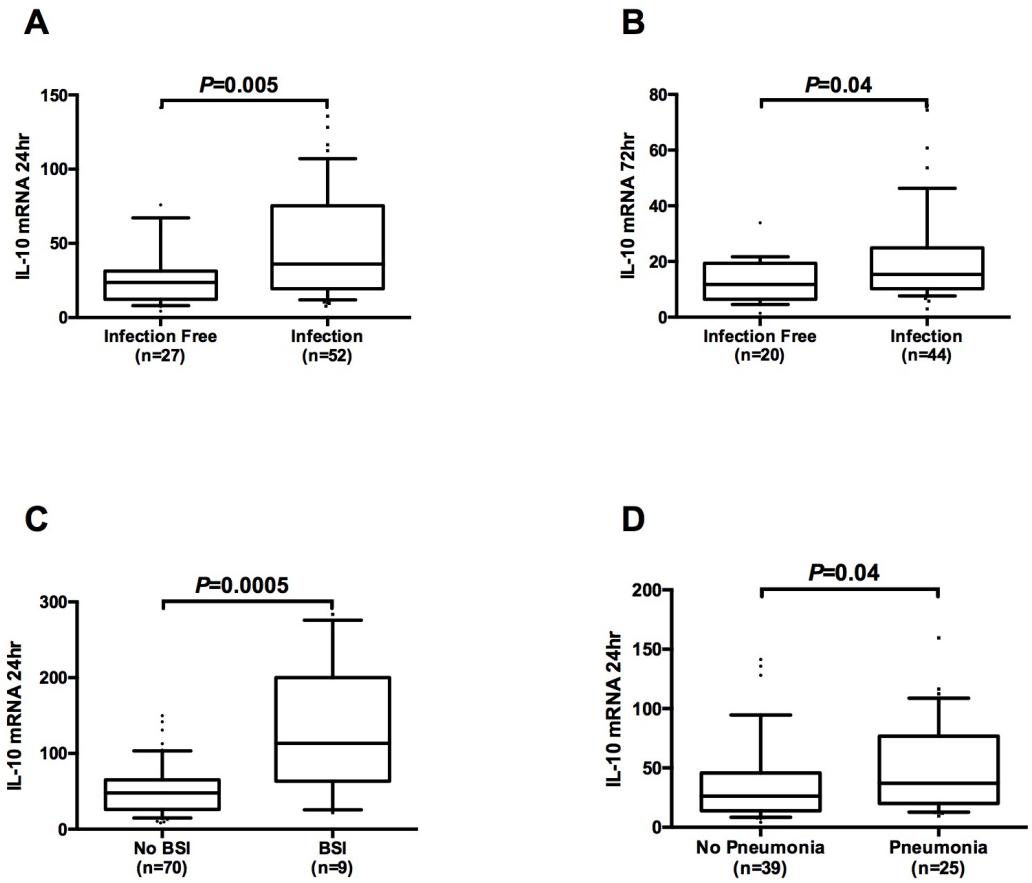


Figure 3.3 - Associations between changes in mRNA in polytrauma patients & the development of late nosocomial infectious complications

Candidate gene mRNA levels assayed at 24 hours (Graphs A, C, D) and 72 hours (B) and their relationship to all infection (Graphs A, B, D), Blood stream infection (C), or pneumonia (D). Graphs A–D, Median, interquartile range (*box*) and the 10th and 90th percentile (*whiskers*). All results are expressed as a relative quantification ratio between candidate and reference genes.

3.4.5 Cytokine gene expression and outcome

23 (19%) patients died during their hospital stay over the course of this study. The median time to death was 5 days (2-7).

3.4.5.1 Anti-Inflammatory/Treg/T_h2 Cytokines

Higher IL-10 mRNA levels at 24 hours were associated with higher mortality rates ($p<0.0001$, Figure 3.4A) and those patients with the greatest rate of rise of IL-10 mRNA over the initial 24 hours were more likely to die ($p<0.05$, Figure 3.4B). Patients with IL-10 mRNA levels in the highest quartile at 24 hours were 7.6 times more likely to die (95% CI 2.08 - 33.3, $p=0.002$).

3.4.5.2 Pro-Inflammatory/T_h1 Cytokines

Those patients whose IFN-γ mRNA levels fell the most over the initial 24 hours were more likely to die subsequently ($p=0.002$, Figure 3.4C).

Those patients who demonstrated the greatest fall in T-bet mRNA levels over the initial 24 hours were more likely to die subsequently ($p=0.0009$, Figure 3.4D). When divided into quartiles, those patients who were in the top quartile for the greatest fall in T-bet mRNA levels over the first 72 hours were 9 times more likely to die (CI 1.7 – 50, $p=0.001$).

At the 72 hour time point those patients with higher absolute levels of IL-12 mRNA levels were more likely to survive their ICU admission ($p=0.004$) and those patients with greatest decreases in IL-12 mRNA over the initial 72 hours ($p=0.005$) were more likely to die (figure 3.4E, F).

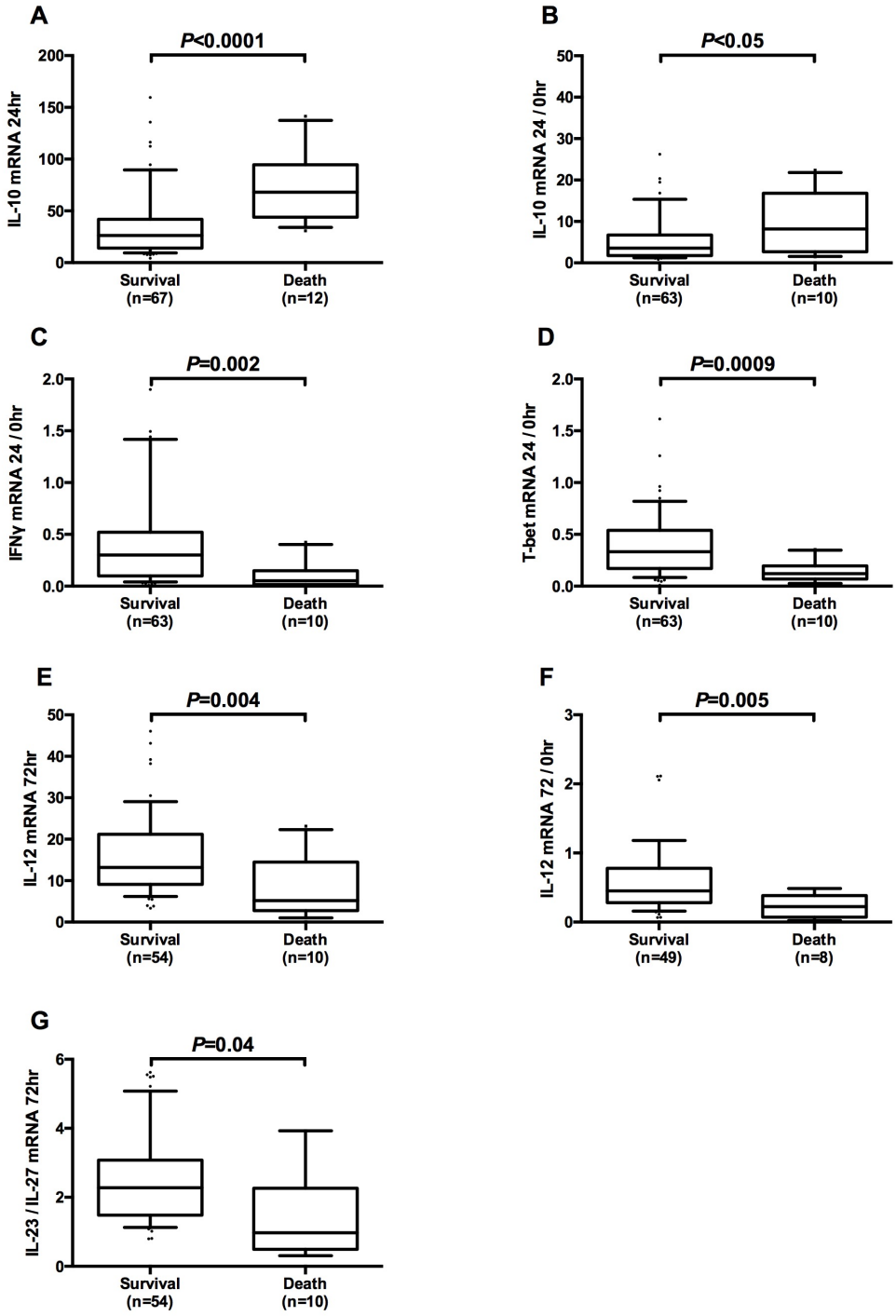


Figure 3.4 - Associations between changes in mRNA in polytrauma patients & survival to hospital discharge

Candidate gene mRNA levels assayed at 24 hours (A), 24 / 0 hours (B-D), 72 hours (E, G), 72 / 0 hours (F) and their relationship to all outcomes (A-G) was calculated. Graphs A–G, Median, interquartile range (*box*) and the 10th and 90th percentile (*whiskers*). All results are expressed as a relative quantification ratio between candidate and reference genes.

3.4.5.3 T_h17 Cytokines

At 72 hours an increased ratio of IL-23/IL-27 was associated with survival to ICU discharge (p=0.04, Figure 3.4G).

3.4.6 Immediate Transfusion Of Blood Products (Prior To Baseline Blood Sampling)

27 (24%) patients received an immediate blood transfusion. In those patients receiving an immediate transfusion the median number of units of red cells transfused was 1.5 (1-2). Packed red cells were the only blood product transfused at this time. Immediate transfusion was not related to the ISS nor was it more common in those with a penetrating as opposed to a blunt mechanism of injury. There was no association between immediate blood transfusion and subsequent mortality. Those receiving immediate transfusions had a greater base deficit (p<0.0001) and a higher serum lactate (p=0.004; Table 3.4).

3.4.7 Early Transfusion Of Blood Products (Within The First 24 Hours)

72 (64%) patients received an early transfusion. All patients who received packed red cells as an early transfusion also received a quantity of fresh frozen plasma (FFP), cryoprecipitate and platelets due to the protocolised nature of early transfusion in this institution (Supplementary Protocols 2 and 3). In those patients requiring early transfusion the median number of units of red cells transfused was 6 (interquartile range 4-10, maximum 53 units), the median number of units of FFP was 4 (1.75-8), and the median number of pools of cryoprecipitate and platelets was 0.5 (0-2) and 1 (0-2) respectively. 21 (19%) patients received a massive transfusion (≥10 units/24 hours) within the first 24 hours.

Those patients requiring early transfusion had a higher ISS (p=0.0003), a greater base deficit (p<0.0001) and a higher serum lactate (p=0.0002). They were also less likely to survive to hospital discharge (p=0.008). The number of units transfused was unrelated to the ISS and

there was no association between a penetrating or blunt mechanism of injury and the requirement for early transfusion (Table 3.5).

3.4.8 Transfusion Of Blood Products & Infectious Complications

There was an association between immediate transfusion and fungaemia (p=0.01; Table 5.2). Of the 40 patients who did not receive an early transfusion one developed a blood stream infection whilst of the 72 patients who did receive an early blood transfusion 15 subsequently developed a blood stream infection (OR 10.3 (1.3-81) p=0.008; Table 3.6). The mean time to the development of a blood stream infection was 8 days. There was no association between ISS and the development of blood stream infection. The organisms isolated are shown in Supplementary Table 7. The incidence of nosocomial infection at other sites is shown in Tables 3.4 and 3.5.

3.4.9 Immediate Administration Of Other Fluids

61 (54%) patients received a median of 500mls (250-750mls) of crystalloid (sodium chloride 0.9%) prior to baseline blood sampling. Immediate administration of crystalloid was associated with higher TNF-α (p=0.02), IFN-γ (p=0.0001) and IL-10 (p=0.01) mRNA at baseline. These associations did not persist in the multivariate models. No association was detected between immediate transfusion of crystalloid and either infectious complications or mortality.

15 (13%) patients received immediate hypertonic saline (sodium chloride 5%). These patients were more likely to die (p=0.03). No association was detected between immediate hypertonic saline administration and infectious complications or any of the baseline candidate gene mRNA levels.

No other intravenous fluid therapy or blood product was administered prior to the baseline blood sampling.

Table 3.4 - Patient characteristics of those suffering polytrauma at 0 hours

	Immediate transfusion *(n=27)*	No immediate transfusion *(n=85)*	*p*-value
Age	41 (30-53)	39 (28.5-57)	*Ns*
Male Sex	20 (74.1%)	69 (81.2%)	*Ns*
ISS	30 (24-38)	29 (20-34)	*Ns*
TBI	8 (29.6%)	48 (56.5%)	0.01
Blunt Injury	24 (92.3%)	75 (89.3%)	*Ns*
Penetrating Injury	2 (7.7%)	9 (10.7%)	*Ns*
Base Deficit	7.9 (4.8-14.1)	3.5 (1.6-5.7)	<0.0001
Lactate	4.9 (2.7-7.1)	2.9 (1.3-4.2)	0.004
PRBC	1.5 (1-2)	0 (0-0)	<0.0001
MOD	14 (51.9%)	29 (34.1%)	*Ns*
ARDS	14 (51.9%)	22 (25.9%)	0.014
All nosocomial Infections	19 (70.4%)	49 (57.7%)	*Ns*
Pneumonia	11 (40.7%)	27 (31.8%)	*Ns*
Bacteraemia	4 (14.8%)	10 (11.8%)	*Ns*
Fungaemia	2 (7.4%)	0 (0%)	0.01
BSI	6 (22.2%)	10 (11.8%)	*Ns*
Wound Infection	4 (14.8%)	17 (20.2%)	*Ns*
UTI	5 (18.5%)	6 (7.1%)	*Ns*
Mortality at 24hr	0 (0%)	2 (2.4%)	*Ns*
Survival to hospital discharge	20 (74.1%)	72 (84.7%)	*Ns*

Data are expressed as median and interquartile range or absolute counts with percentages in parenthesis. *AIS*, Abbreviated injury score. *ARDS*, Acute respiratory distress syndrome: This was defined using the respiratory component of the daily sequential organ failure assessment (SOFA) scores and ARDS was deemed present if the respiratory component of the SOFA score was ≥3 for 2 consecutive days. *BSI*, blood stream infection (encompassing bacteraemia and fungaemia). *ISS*, injury severities score. *Ns,* non-significant. *MOD*, multiple organ dysfunction: This was defined, using the SOFA score, as the presence of ≥2 organs from the SOFA score being ≥3 in 24 hours. *PRBC*, packed red blood cells. *TBI*, traumatic brain injury. *UTI*, urinary tract infection.

3.4.10 Early Administration Of Other Fluids

109 (98%) patients received a median of 2920mls (2000-4600mls) of crystalloid (sodium chloride 0.9% or Plasma-Lyte) over the first 24 hours. The volume of crystalloid administered was associated with the ISS ($r^2 = 0.04$, p=0.03) and with blood stream infections (p=0.03), but not with mortality. No association was detected between early crystalloid administration and any of the candidate gene mRNA levels.

22 (20%) patients received a median of 700mls (462-775mls) of hypertonic saline (sodium chloride 5%) in the first 24 hours. These patients had a higher ISS (p=0.01) and were more likely to die (p=0.02). Early administration of hypertonic saline was associated with higher TNF-α (p=0.0008), IL-12 (p=0.005), RORγt (p=0.03) and FOXP3 (p=0.003) mRNA at 24 hours. On multivariate analysis the only independent association that remained was between higher TNF-α mRNA levels at 24 hours and the volume of hypertonic saline administered in the first 24 hours.

87 (78%) patients received a median of 1000mls (455-2000mls) colloid (Geloplasma®) in the first 24 hours. The volume of colloid was related to the ISS ($r^2 = 0.05$, p=0.01) and larger volumes of colloid were associated with higher mortality (p=0.001). Larger volumes of colloid were associated with higher IL-10 mRNA levels at 24 hours ($r^2 = 0.12$, p=0.002). On multivariate analysis there was no independent association between IL-10 mRNA levels and colloid administration.

No other intravenous fluid therapy or blood product was administered during the initial 24 hours in these patients.

Table 3.5 - Patient characteristics of those suffering polytrauma at 24 hours

	Transfused in 1st 24 hours *(n=72)*	**No transfusion in 1st 24 hours** *(n=40)*	***p*-value**
Age	39.5 (30-57.8)	43 (27-57)	*Ns*
Male Sex	57 (79.2%)	32 (80%)	*Ns*
ISS	30 (22.5-41)	22 (17.5-32.8)	0.0003
Head Injury	28 (38.9%)	28 (70%)	0.002
Blunt Injury	62 (87.3%)	37 (94.9%)	*Ns*
Penetrating Injury	9 (12.7%)	2 (5.1%)	*Ns*
Base Deficit (0hr)	5.8 (2.6-9.6)	2 (0.6-4.3)	<0.0001
Lactate (0hr)	3.4 (2.45-6.05)	1.8 (1-3.6)	0.0002
PRBC	6.5 (4-10)	0 (0-0)	<0.0001
FFP	4 (0.4-8)	0 (0-0)	<0.0001
PLT	1 (0-2)	0 (0-0)	<0.0001
Cryoprecipitate	0.5 (0-2)	0 (0-0)	<0.0001
MOD	31 (43.1%)	12 (30%)	*Ns*
ARDS	29 (40.3%)	7 (17.5%)	0.02
All nosocomial Infections	47 (65.3%)	21 (52.5%)	*Ns*
Pneumonia	25 (35.2%)	12 (28.2%)	*Ns*
Bacteraemia	13 (18.1%)	1 (2.5%)	0.017
Fungaemia	2 (2.8%)	0 (0%)	*Ns*
BSI	15 (20.8%)	1 (2.5%)	0.008
Wound Infection	18 (25%)	4 (10%)	*Ns*
UTI	7 (9.7%)	4 (10%)	*Ns*
Mortality at 24hr	2 (2.8%)	0 (0%)	*Ns*
Survival to hospital discharge	54 (75%)	38 (95%)	0.008

Data are expressed as median and interquartile range or absolute counts with percentages in parenthesis. *AIS*, Abbreviated injury score. *ARDS*, Acute respiratory distress syndrome: This was defined using the respiratory component of the daily sequential organ failure assessment (SOFA) scores and ARDS was deemed present if the respiratory component of the SOFA score was ≥3 for 2 consecutive days. *BSI*, blood stream infection (encompassing bacteraemia and fungaemia). *FFP*, fresh frozen plasma. *ISS*, injury severities score.. *MOD*, multiple organ dysfunction: This was defined, using the SOFA score, as the presence of ≥2 organs from the SOFA score being ≥3 in 24 hours. *Ns,* non-significant. *PLT*, platelets. *PRBC*, packed red blood cells. *TBI*, traumatic brain injury. *UTI*, urinary tract infection. Patients who survived were either discharged to a rehabilitation facility.

3.4.11 Univariate Analysis Of The Association Between Blood Transfusion & Gene Expression

3.4.11.1 Anti-Inflammatory Mediators

IL-10 (p=0.003; figure 3.5A) and IL-27 (p=0.04; figure 3.5B) mRNA levels were higher at 0-hour in those patients who had received an immediate blood transfusion. IL-10 mRNA levels rose significantly over the initial 24 hours in all patients (p<0.0001) but to a greater extent in those receiving an early transfusion (p=0.01). At 24 hours IL-10 mRNA levels were higher in those patients who received early transfusions (p<0.0001; figure 3.5C). Similarly, there was a significant positive correlation between the number of red cells transfused and IL-10 mRNA levels at 24 hours (Spearman's ρ 0.61, p<0.0001; figure 3.5D).

FOXP3 mRNA levels at 24 hours were lower in those patients receiving an early transfusion (p=0.01). The number of units of packed red cells transfused was inversely correlated with FOXP3 mRNA levels (Spearman's ρ -0.39, p=0.0004). Those patients who received an early transfusion had lower GATA-3 mRNA levels (p=0.006) at 24 hours.

There was no association between transfusion and gene expression of anti-inflammatory mediators at 72 hours.

3.4.11.2 Pro-Inflammatory T_h1 Mediators

In the group as a whole, TNF-α mRNA levels rose from baseline to 24 hours. This increase was less in those patients who received an early transfusion (p=0.015; figure 3.6A). Greater reductions in IFN-γ mRNA (p=0.035; figure 3.6B) and T-bet mRNA (p=0.03; figure 3.6C) over the first 24 hours were seen in those patients receiving an early transfusion. There was no association between transfusion and gene expression of T_h1 mediators at 72 hours.

3.4.11.3 Pro-Inflammatory T_h17 Mediators

RORyt mRNA levels at 24 hours (p=0.05; figure 3.7A) and 72 hours (p=0.02; figure 3.7B) were lower in those patients who received early transfusion.

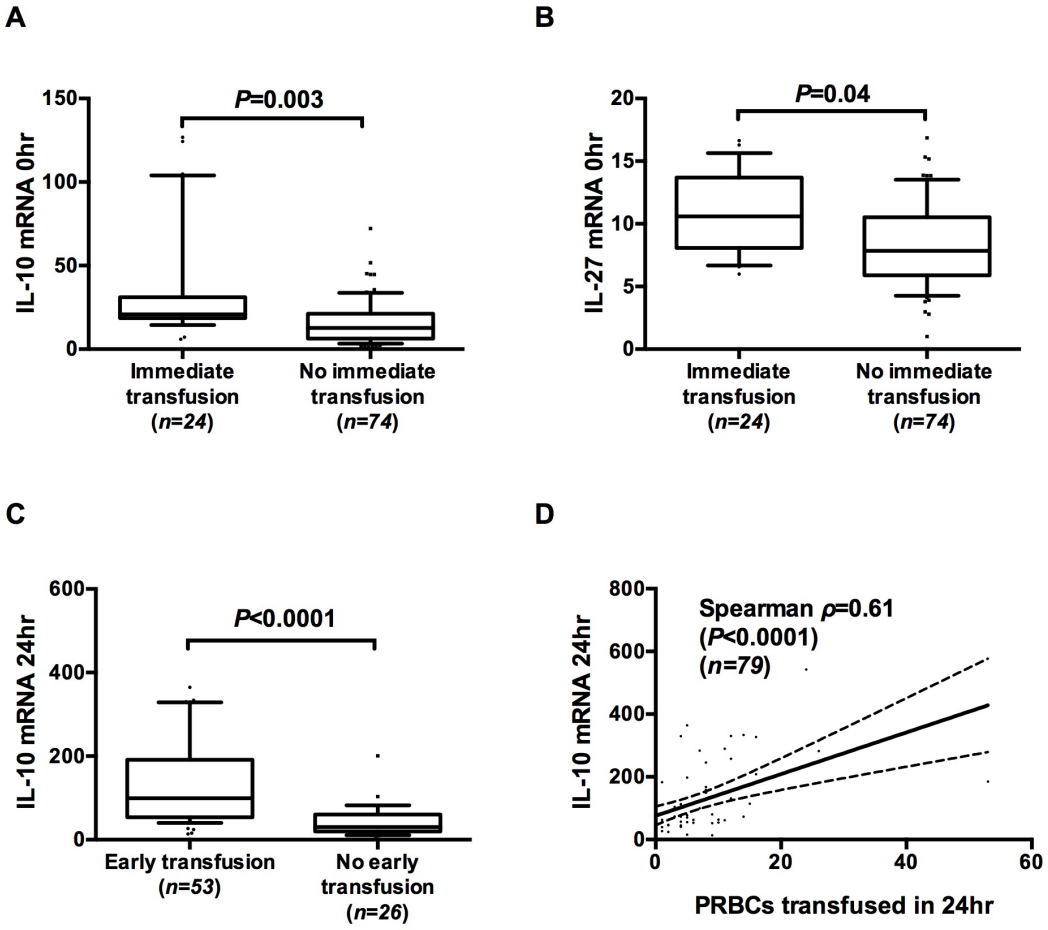


Figure 3.5 - Associations between anti-inflammatory mRNA levels & blood transfusion following polytrauma

Graphs A&B candidate gene mRNA levels at 2 hours following polytrauma and immediate transfusion of blood products. Graphs C&D candidate gene mRNA levels at 24 hours following polytrauma and early transfusion of blood products. (A-C) Median, interquartile range (*box*) and the 10th and 90th percentile (*whiskers*). (D) Association between the number of units of packed red blood cells (PRBCs) transfused and candidate gene mRNA levels at 24 hours (best-fit line (*solid*) and 95% confidence interval (*dashed*)). All results are expressed as a relative quantification ratio between candidate and reference genes.

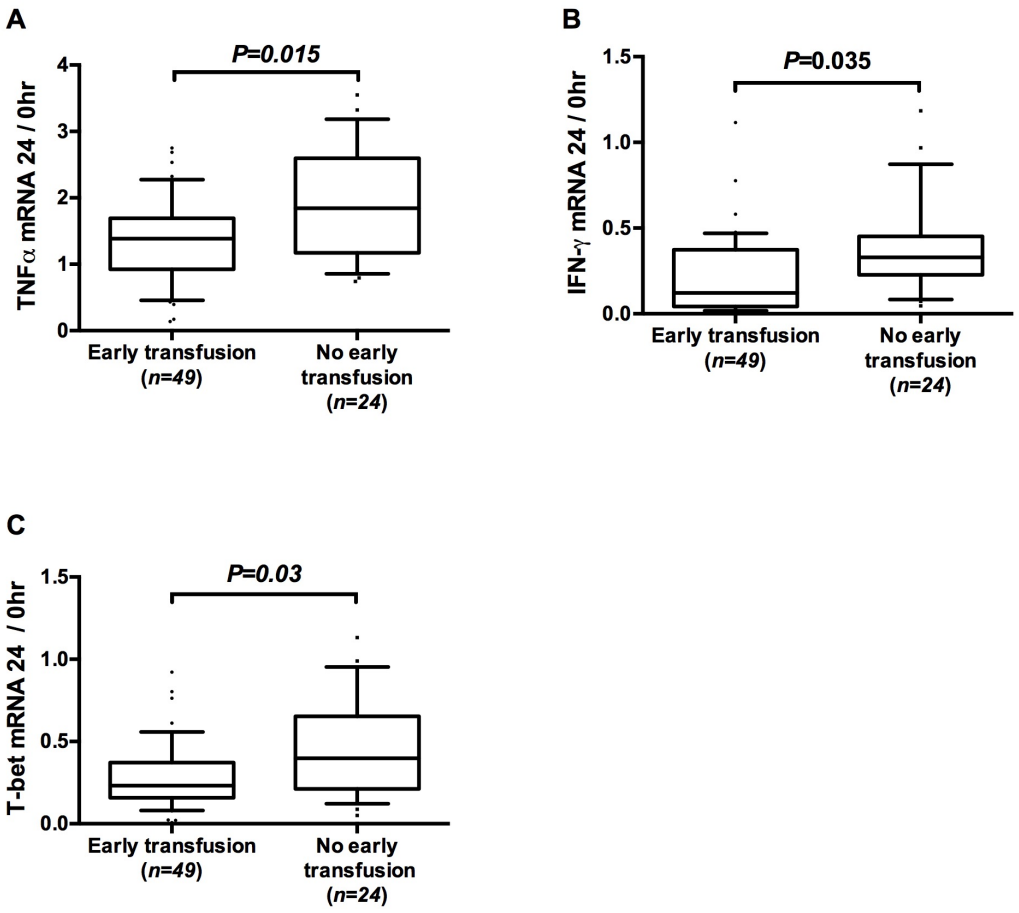


Figure 3.6 - Associations between T_h1 related mRNA levels & blood transfusion in the first 24 hours following polytrauma

Ratio of candidate gene mRNA levels at 24 hours to levels at baseline (within 2 hours of the trauma) in relation to the transfusion of blood products over the first 24 hours (median, interquartile range (*box*) and the 10^{th} and 90^{th} percentile (*whiskers*)). All results are expressed as a relative quantification ratio between candidate and reference genes.

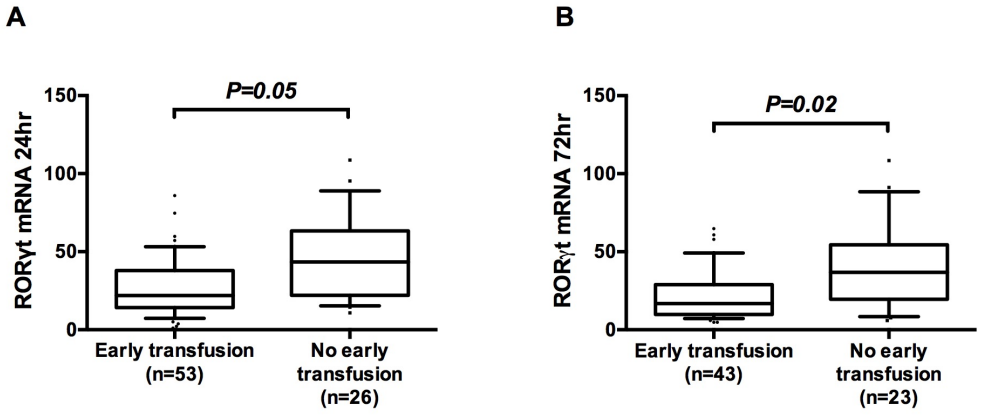


Figure 3.7 - Associations between T_h17 related mRNA & blood transfusion following the first 24 hours of polytrauma

Candidate gene mRNA levels at 24 hours (A) and 72 hours (B) following polytrauma and early transfusion of blood products (median, interquartile range (*box*) and the 10th and 90th percentile (*whiskers*)). All results are expressed as a relative quantification ratio between candidate and reference genes.

3.4.12 Multivariate Analysis Of The Influence of Allogeneic Transfusion

The results and selection of variables included in the univariate analyses are shown in Supplementary Tables 5 & 6.

The requirement for immediate transfusion remained independently associated with IL-10 mRNA at time 0 (p=0.04). IL-27 mRNA at time 0 was not entered into the multivariate analysis because, apart from immediate transfusion, none of the associations on univariate analysis achieved a p-value of <0.1.

At 24 hours there was an independent association between the number of units of red cells transfused and IL-10 mRNA levels (p<0.0001), following backwards elimination.

There was an independent association between a lesser increase in TNF-α mRNA over the initial 24 hours (24 hours / 0 hours) and the requirement for early transfusion (p=0.05). Following backwards elimination the requirement for early transfusion was independently

associated with the magnitude of the decrease in IFN-γ mRNA over the initial 24 hours (p=0.006). Early transfusion was not independently associated with RORγt mRNA levels at 24 hours in this model (Supplementary Table 6).

3.4.13 Age Of Blood Analysis Cohort

From this group data were available for 64 patients who received a PRBC transfusion within 24 hours of admission. 53 (83%) patients were male and median patient age was 40.5 years (IQR 31-59). Median ISS was 31.5 (IQR 23-43) and 55 (86%) patients suffered blunt injury. Median base deficit on admission was 4.9 mEq/L (IQR 2.4-8.8) and at 24 hours was 0.7 mEq/L (IQR -0.7-2.6). 40 (63%) patients developed infectious complications and 15 (23%) patients died before hospital discharge.

Each patient received a median of 5 units (IQR 4-9.8) of PRBCs over the first 24 hours of hospital admission. Median age of the transfused PRBCs for each patient was 20 days (IQR 17-22). Patients also received a median of 4 units (IQR 0-8) fresh frozen plasma (FFP) and 1 unit (IQR 0-1) of platelets. Those patients receiving in excess of 35 units of PRBCs were excluded from the analysis (n=8). The Characteristics of the cohort are fully outlined in Table 3.6.

3.4.14 Univariate Analysis Of Age Of PRBCs Transfused & Gene Expression

At baseline (blood sample within 2 hours of the trauma) there was an association between FOXP3 mRNA levels and the median age of the transfused PRBCs (r^2=0.18, p=0.001) and also between GATA-3 mRNA levels and the median age of the transfused PRBCs (r^2=0.09, p=0.03). No other associations were detected between gene expression at baseline and the median age of the PRBCs.

Change in gene expression was expressed by taking a ratio of both the 24 hour or the 72 hour mRNA level to the baseline mRNA level. There was an association between

decreasing IL-12 (r^2=0.10, p=0.03), IL-23 (r^2=0.10, p=0.03), RORγt (r^2=0.19, p=0.003), FOXP3 (r^2=0.09, p=0.04) and GATA-3 (r^2=0.16, p=0.007) mRNA over 24 hours and increasing age of the transfused PRBCs.

Table 3.6 - Patient characteristics of those polytrauma patients transfused in the first 24hr

	Median Age of Blood Transfused		***p*-value**
	1-20 days (n=38)*	*21-35 days* (n=26)*	
Male	33 (86.8)	20 (76.9)	*Ns*
ISS	34 (25 - 48)	27 (22 - 38)	*Ns*
Presence of TBI	17 (44.7)	10 (38.5)	*Ns*
Blunt Injury	33 (86.8)	22 (84.6)	*Ns*
Penetrating Injury	5 (13.2)	3 (11.5)	*Ns*
On Scene *s*BP	112 (90 - 135)	115 (94 - 130)	*Ns*
Emergency Department *s*BP	106 (80 - 139)	114 (93 - 120)	*Ns*
Base deficit (0hr)	4.7 (2.6 - 7.5)	5.2 (2.4 - 14.1)	*Ns*
Lactate (0hr)	2.9 (2.1 - 4.5)	3.5 (2.2 - 6.8)	*Ns*
pH (0hr)	7.29 (7.20 - 7.36)	7.29 (7.14 - 7.40)	*Ns*
Base deficit (24hr)	0.45 (-0.7 - 1.43)	2.4 (-1.3 - 3.2)	*Ns*
Number of PRBCs transfused	7 (4 - 10)	5 (4 - 8)	*Ns*
Massive Transfusion (≥10 units/24hrs)	6 (16.0)	5 (19.2)	*Ns*
All nosocomial Infections	24 (63.2)	16 (62.0)	*Ns*
Pneumonia	13 (34.2)	9 (35.0)	*Ns*
BSI	8 (21.1)	4 (15.4)	*Ns*
Wound Infection	11 (29.0)	4 (15.4)	*Ns*
UTI	5 (13.2)	1 (3.8)	*Ns*
Survival To Hospital Discharge	30 (79.0)	19 (73.1)	*Ns*

*Data are expressed as median (interquartile range) or n (%). ISS, Injury Severity Score; TBI, Traumatic Brain Injury; sBP, Systolic Blood Pressure; PRBCs, Packed Red Blood Cells; BSI, Blood stream Infection (bacteraemia or fungaemia); UTI, Urinary Track Infection. *Patients divided into two groups based on median age of PRBCs transfused in first 24 hours; ≤20 days vs. >21 days.*

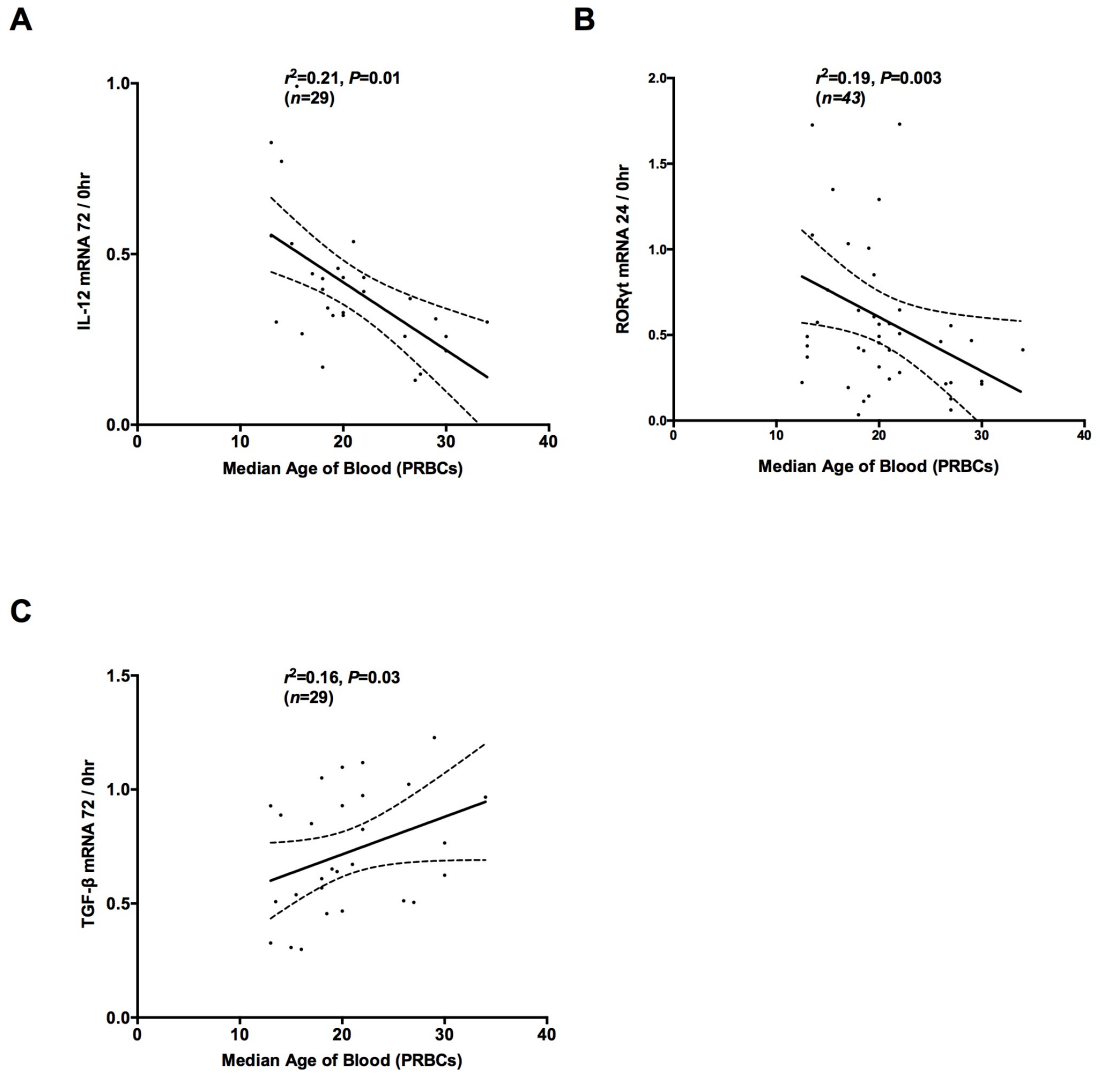


Figure 3.8 - Associations between the median age of PRBCs transfused in the first 24 hours following injury & the change in candidate gene expression

Over 72 hours (A and B) and 24 hours (C). Results displayed as best-fit line (solid) and 95% confidence interval (dashed). All results are expressed as a relative quantification ratio between candidate and reference genes.

There was an association between decreasing IL-12 mRNA over 72 hours (r^2=0.20, p=0.01) and increasing age of the transfused PRBCs. There was also an association between increasing TGF-β mRNA over 72 hours (r^2=0.16, p=0.03) and increasing age of the transfused PRBCs.

No associations were detected between changes in IL-10, TNF-α, IFN-γ, T-bet, and IL-27 gene expression and the age of the transfused PRBCs.

3.4.15 Multivariate Analysis Of Age Of PRBCs Transfused & Gene Expression

Multivariate linear regression models were created to test for independent associations between change in gene expression and the median age of transfused PRBCs in cases where a univariate association was detected. The selection of predictor variables is outlined in the methods and presented in Supplementary Table 8.

For decreasing IL-12 over 24 and 72 hours, decreasing RORγt over 24 hours and increasing TGFβ over 72 hours an independent association between change in gene expression and the median age of the PRBCs transfused was also present (Supplementary Table 8). In the case of decreasing FOXP3, GATA-3 and IL-23 over 24 hours the variation in gene expression levels was better explained by variations in patient age as opposed to variations in the median age of the PRBCs (Supplementary Table 8). Supplementary Table 9 demonstrates the model, and model performance with all predictor covariates included.

3.4.16 Age of PRBCs and infections

No associations were detected between the age of PRBCs transfused and nosocomial infections or in-hospital mortality.

3.5 Discussion

Here we present data demonstrating that a well-developed, directional inflammatory response is present within 2 hours of severe injury. The very early elevation of the prototypical anti-inflammatory cytokine IL-10 attests to the immunosuppressive nature of this initial response. Immediate reduced activity in the pro-inflammatory T_h17 pathway, as evidenced by falling levels of the T_h17 specific transcription factor, RORγt, and an avid T_h17 promoting

cytokine, IL-23, further adds to this pattern. There is an immediate rise in levels of the T_h1 effector cytokine (IFN-γ), however levels of this cytokine fall below the control cohort after 24 and 72 hours. The pro-inflammatory T_h1 pathway demonstrates a suppressed pattern over the initial 24 hours with falling levels of promoters of the T_h1 pathway (IL-12) and T_h1 specific transcription factors (T-bet). In addition, TNF-α levels immediately fall compared to the control cohort. These values rise at 24 hours, however still remaining below the assayed healthy control cohort, staying static at 72 hours. This rapid, predominantly anti-inflammatory response to trauma is largely unexpected in the context of previous publications (Mannick *et al.*, 2001; Xiao *et al.*, 2011). It is very significant that the magnitude of the early anti-inflammatory response or the failure to mount or maintain a pro-inflammatory response is closely associated with the acquisition of subsequent infections and also with death.

Utilising this same panel of interlinked cytokines and transcription factors, the immunomodulatory effects of the transfusion of blood and blood products in patients with severe traumatic injury was also investigated. This demonstrated that the immunosuppressive nature of the acute traumatic immune response is compounded by the transfusion of allogeneic blood. This is again characterised by the dramatic and immediate up-regulation in gene expression of the archetypal anti-inflammatory gene, IL-10, while a concurrent immediate rise in IL-27, a gene known to vigorously suppress the pro-inflammatory T_h17 pathway supports this concept (Hunter & Kastelein, 2012). This is followed by a decrease in RORγt, which is further evidence for a repressed T_h17 activity associated with blood transfusion in trauma patients. Although developing at a slightly slower pace, the pro-inflammatory T_h1 pathway demonstrates a reciprocal down-regulation, with greater falls in IFN-γ, and the T_h1 specific transcription factor, T-bet, in those patients receiving blood products in the first 24 hours following severe trauma. Although TNF-α gene expression increased in the cohort of trauma patients as a whole over the initial 24 hours; the rate of rise was reduced in those patients receiving early transfusion of blood products. These alterations then become more pronounced and extensive over the initial 3 days following ICU admission. Those patients transfused had an

increased severity of injury and shock, as well as an increased incidence of nosocomial infection and an increased risk of death.

A further analysis investigated the relationship between the duration of storage of PRBCs prior to transfusion and the resultant immunomodulation. We identified that a pattern of altered gene expression consistent with greater immunosuppression is associated with the transfusion of older PRBCs. This showed reductions in the activity in the pro-inflammatory pathways as evidenced by reduced gene expression of the prototypical T_h1 polarising cytokine, IL-12, as well as those involved in T_h17 function, IL-23, and RORγt. The increased expression of the apoptotic and anti-inflammatory gene, TGF-β, with increasing age of transfused PRBCs further points to the immunosuppressive nature of the response.

Whilst it has been accepted that severe traumatic injury leads to swift alterations (<12 hours) in gene expression in up to 80% of the total genome, precisely how rapidly this occurs has yet to be defined (Xiao *et al.*, 2011). Also lacking to date has been a robust analysis of the time course of changes in gene expression of key cytokines and how these relate to important clinical variables. To further investigate these issues reliably an appropriate choice of laboratory technique is essential.

Available methodologies include analysis of the protein product and whole genome gene expression techniques. Protein analysis, however, has proven to lack sensitivity *in vivo* and has consistently failed to detect multiple target proteins both in trauma studies and in studies of ICU sepsis (Gouel-Cheron *et al.*, 2012; O'Dwyer *et al.*, 2006). It is interesting that previously IL-10 protein was specifically found to be undetectable in the peripheral blood of severely injured trauma patients despite other laboratory evidence of overt immunoparalysis (Gouel-Cheron *et al.*, 2012).

Whilst genome wide gene expression studies provide a powerful overview of alterations to the transcriptome as a whole this approach necessarily has a reduced sensitivity in

the analysis of subtle changes in the expression levels of individual genes. In contrast, quantification of mRNA levels of key cytokines using PCR technologies has proven accurate as a measure of the strength and direction of the immune response when the candidate genes are carefully selected to represent specific immune pathways (O'Dwyer *et al.*, 2006; O'Dwyer *et al.*, 2008; Pachot *et al.*, 2005). Furthermore, the additional analysis of transcription factors specific for T cell subtypes allows inferences to be made as regards the balance of activity between competing T helper cell subtypes and to elucidate potential cellular sources of cytokines of interest (Pachot *et al.*, 2005).

Utilising this technique we observed a 6 fold increase in IL-10 gene expression occurring within 2 hours of major trauma. Levels continue to increase dramatically over the initial 24 hours, peaking prior to day three. To date, sampling limitations have resulted in the best estimate of an immunological response to trauma as occurring initially between 4-12 hours (Xiao *et al.*, 2011). The source of this excess IL-10 remains unclear from our data. As levels of the T_{reg} cell specific transcription factor FOXP3 and the T_h2 specific transcription factor GATA-3 both fall in response to severe trauma these T cell subtypes are unlikely to contribute to the observed excess IL-10 gene expression. This is surprising given that T_{reg} cells are thought to increase, both as a proportion of total T lymphocytes and as absolute counts, following trauma, due in part to their resistance to apoptosis (Venet *et al.*, 2008). It seems clear, however, that FOXP3 expression must increase in the presence of enhanced natural T_{reg} cell activity and therefore the fall in FOXP3 mRNA probably indicates lowered natural T_{reg} cell activity in our cohort (Campbell & Ziegler, 2007). Alternative sources of IL-10 are abundant amongst innate and adaptive cell types and include dendritic cells, macrophages, mast cells, eosinophils, neutrophils, $CD4^+$ and $CD8^+$ T cells, B cells as well as release from damaged muscle or from the endothelium (Ouyang *et al.*, 2011). It is also interesting that we found only relatively modest associations between IL-10 levels and the severity of injury, which indicates a large amount of inter-individual variability in IL-10 gene expression. It is interesting to postulate that genetic or epigenetic factors may account for a degree of the unexplained variability (Owen *et*

al., 2014). By normalising candidate gene PCR expression data to reference genes which were demonstrated to be expressed at stable levels in these patients (ATP5B and GAPDH) we were able to correct for the effect of relative changes in the total leukocyte population in individual whole blood samples (Vandesompele *et al.*, 2002). We cannot, however exclude the possibility that the observed changes in mRNA levels are explained, at least in part, by alterations in the relative abundance of specific leucocyte subpopulations collected in the whole blood samples from which the RNA was extracted for this thesis (Laudanski *et al.*, 2006). It is also plausible that small variations in the qRT-PCR assay could have led to changes in the amplified signal despite having made every effort to standardise the process; (templates (primers and probes), mRNA (concentrations and RNA integrity), the reverse transcription step and reference genes) as well as a single operator (HDTT).

IL-6 is a notable omission from our panel of cytokines that is deserving of further explanation. Recently, IL-6 protein levels following trauma were found to be increased (Gouel-Cheron *et al.*, 2012). However, the exact role of IL-6 in this scenario is unclear. Although commonly considered a classical pro-inflammatory cytokine, IL-6 is also known to exert strong anti-inflammatory effects by promoting T_h2 differentiation and inhibiting T_h1 polarisation (Diehl & Rincon, 2002). Indeed, in the setting of severe injury higher initial IL-6 levels were associated with a propensity to develop infectious complications later (Gouel-Cheron *et al.*, 2012). Consequently, we omitted this cytokine from our analysis as we found it difficult to ascribe to it an unambiguous inflammatory direction in the setting of trauma.

An early, dominant anti-inflammatory response would contradict paradigms of inflammation, which describe either a sequential SIRS and CARS response (Mannick *et al.*, 2001; Moore *et al.*, 1996) or parallel SIRS and CARS responses to severe injury (Xiao *et al.*, 2011). However, a review of the data, which utilise cytokine quantification to support the presence of a dominant early SIRS response following trauma, finds that the conclusions rely heavily on animal models and on the unbalanced quantification of small groups of pro-

inflammatory cytokines (Marik & Flemmer, 2012). In contrast, there is an abundance of descriptions of lymphocyte anergy and apoptosis and impaired monocyte phagocytosis and antigen presentation in response to trauma (Cheron *et al.*, 2010; Faist *et al.*, 1988; Faist *et al.*, 1996; Gouel-Cheron *et al.*, 2012; Kampalath *et al.*, 2003; Tschoeke & Ertel, 2007). It is plausible then that this paradigm requires further revision with lesser emphasis on the systemic pro-inflammatory response to severe injury. Interestingly, the inflammatory paradigm is also being reevaluated in other scenarios where traditionally a pro-inflammatory state was considered dominant. Ancillary treatment of ICU sepsis has progressed from futile attempts to blunt the host immune response to more promising therapies with immunostimulants (Fisher *et al.*, 1996; Meisel *et al.*, 2009; Sprung *et al.*, 2008). Similarly, although it has been a commonly held belief that endurance exercise induces a SIRS type response, analysis of cytokine levels demonstrates a marked rise in IL-10 following an exercise protocol with no immediate rise in TNF-α observed (Pournot *et al.*, 2011).

In those patients with less severe injuries it may be plausible to suggest that elevated IL-10 levels in peripheral blood are the systemic manifestations of a CARS response to a localised injury which itself has initially induced a compartmentalised SIRS pro-inflammatory response. However, as the vast majority of the patients recruited to this study suffered very severe polytrauma, as distinct from a minor discrete injury, any pro-inflammatory response should be easily detectable systemically and not localised to a specific tissue bed. It was surprising therefore that key markers of innate immunity and activity of the pro-inflammatory T_h1 and T_h17 pathways, aside from the admission rise of IFN-γ, were reduced by trauma over the course of three days of sampling.

A developing anti-inflammatory profile has been associated with an increased susceptibility to develop infectious complications both following severe trauma and in the perioperative period (Cheron *et al.*, 2010; White *et al.*, 2011). Similarly, we hypothesised that the increased IL-10 gene expression we observed may increase susceptibility to infectious

complications, particularly in the setting of reduced pro-inflammatory, bactericidal cytokine gene expression. In that context, the association between late infections and excess IL-10 mRNA is unsurprising and may be very significant in terms of potential novel immunomodulating treatments. It is notable that the tendency to develop definitive blood stream infections was particularly strongly associated with excess IL-10 production. Manipulating the immune response of trauma patients has previously met with mixed results (Spruijt *et al.*, 2010). However, the lack of robust diagnostics to characterise a group, which may derive maximum benefit coupled with late administration of the treatment, may account for the disappointing results. Whilst previously described associations between trauma induced immunosuppression and subsequent infections require repeated assessment of HLA-DR expression up to the fourth day following trauma our data demonstrates an association at 24 hours between excess IL-10 and subsequent infection (Cheron *et al.*, 2010). It is notable that in critically ill septic patients IL-10 is partially responsible for the proposed endocytosis of HLA-DR molecules in monocytes, which is a characteristic of the septic immunoparalysed patient (Fumeaux & Pugin, 2002). A similar mechanism following severe injury may help explain the apparent association between excess IL-10, reduced cell wall HLA-DR and the susceptibility to develop infectious complications.

The association between early gene expression and outcome is quite striking. Those patients identified as having the greatest IL-10 production have a 7.6 fold increased risk of death. Reduced activity of pro-inflammatory pathways is equally associated with poor outcome. Whether these associations invoke causation or are an epiphenomenon remains unclear at present. One could hypothesise that excess IL-10 and a relative deficiency of pro-inflammatory compounds leads to an enhanced susceptibility to develop infectious complications, which in turn leads to greater mortality. However, most deaths in our patient group occurred relatively early and as a consequence we failed to describe an association between infection and mortality. When early deaths were excluded from the analysis then a slightly greater number of patients developing infection subsequently died. Others have

described a significant association between sepsis and death following trauma in a much larger cohort of 30,000 patients, which included patients with milder injuries (Osborn *et al.*, 2004). Furthermore, if the excess IL-10 produced is more than just an epiphenomenon then it plausibly has more diverse detrimental effects than simply promoting immunoparalysis following trauma.

It is difficult to understand the advantage accruing to an organism from an anti-inflammatory response to severe trauma. Although the associations between an anti-inflammatory cytokine profile and blood transfusion are interesting the study is not powered to derive firm conclusions from these data. Perhaps the cells producing IL-10 are primarily concerned with tissue repair and systemic immunoparalysis is an unintended effect. Furthermore, it is a well described strategy of microorganism to evade capture by the host immune response by stimulating the production of IL-10 in dendritic cells (Jang *et al*., 2004; Netea *et al*., 2004) and it is plausible that damage associated molecular patterns (DAMPs) invoke a similar response with no host advantage accruing. Further studies may answer these questions.

What has been less clear in the trauma literature is whether blood transfusion exacerbates this underlying immunosuppressive response to injury described here. The utilisation of multivariate regression analysis indicates that the transfusion of blood products is associated with a more immunosuppressive pattern of gene expression, independent of a number of key variables, which describe the extent of tissue injury, early tissue ischaemia and shock. It is plausible, therefore, that transfusion of blood products exerts a significant direct influence on the pro - / anti - inflammatory balance of the immune response following severe trauma. Allogeneic blood transfusions are well known to possess immunomodulatory properties which are mediated through multiple mechanisms, including the presence of soluble biological response modifiers and the persistence of residual white blood cells despite leukodepletion (Blajchman, 2002; Vamvakas, 2002; Vamvakas & Blajchman, 2007). Older

blood in particular can induce host regulatory T cell (T_{reg}) activation and impair innate immunity, possibly through the direct administration of allogeneic cytokines (Baumgartner *et al.*, 2009a; Baumgartner *et al.*, 2009b; Escobar *et al.*, 2007; Patel *et al.*, 2006).

The best-described practical consequence of the immunosuppression associated with blood transfusion is an enhanced susceptibility to infectious complications. In the perioperative setting blood transfusion has been associated with an increased risk of infections (Cata *et al.*, 2013; Jensen *et al.*, 1992). These findings are not universal however, particularly in patients receiving leukodepleted blood (Cata *et al.*, 2013). An increase in infectious complications has also been noted in trauma patients, although some animal models suggest that it is the haemorrhage itself, which is responsible for exacerbating immunosuppression (Cue *et al.*, 1992; Jackman *et al.*, 2012). The commonest infectious complication reported in trauma patients is pneumonia (Magret *et al.*, 2010). However, pneumonia is a complex end-point in trauma patients, as significant numbers of patients suffer thoracic injuries with associated pulmonary infiltrates, which are frequently misdiagnosed as pneumonia. Using a more robust measure of definitive infection, namely blood stream infection, we report that in our cohort there is an excess of patients developing blood stream infections amongst those receiving a transfusion of blood or blood products. Although there is also an increased mortality amongst those who were transfused, these patients are also more severely injured.

There remains a paucity of data on the time course of the immune response associated with transfusion in trauma patients. However, cytopenic haematology patients have been previously noted to exhibit raised TGFβ levels within an hour of transfusion (Apelseth *et al.*, 2011). A similarly swift alteration in gene expression was noted in the cohort described here. Our blood samples were taken in the emergency department a maximum of 2 hours following the traumatic insult. In many individual cases the changes in gene expression occurred significantly less than 2 hours from the time of blood transfusion.

The pattern of immune response we describe evolves over time and includes an immediate activation of anti-inflammatory pathways followed later by suppression of pro-inflammatory innate, T_h1 and T_h2 pathways. A network of interlinked immune responses may account for this temporal relationship, with the initial rise in IL-10 requiring time to affect pro-inflammatory responses. However, in this cohort all the immediate transfusions (0-2 hours post trauma) consisted solely of packed red cells whilst all of the transfusions required from this time point to 24 hours also included fresh frozen plasma and platelets. It is possible, therefore that the various blood products had differing effects on the immune response. It has been demonstrated, for example, that fresh frozen plasma contains a predominance of T_h2 cytokines, whereas platelet transfusions have been noted to cause an increase in TGF-β levels (Apelseth *et al.*, 2011; Theusinger *et al.*, 2011). It is, however, impossible to differentiate the *in vivo* effects of different blood products in observational studies of trauma victims given their protocolised co-administration in this setting.

The observational data presented in this facet of the Chapter should be viewed as hypothesis generating. The inclusion and exclusion criteria for the ACIT2 study may have created a selection bias resulting in the under-recruitment of rapidly bleeding patients dying acutely of haemorrhagic shock. This may partially account for the low mortality rate observed in the first 24 hours in this cohort and how this potential selection bias may affect the results is unclear. It is possible that unknown confounding variables affecting gene expression were omitted from our multivariate regression analysis, despite the inclusion of three variables descriptive of the severity of shock (on scene blood pressure, initial serum lactate and base deficit) in addition to the ISS. It is also plausible that the requirement for blood transfusion may simply identify those patients with a greater severity of injury more accurately than the ISS and perhaps define tissue ischaemia more accurately than lactate, base excess or blood pressure measurements. Although no distinct pattern of gene expression was independently associated with non-blood based intravenous fluid therapies over the initial 24 hours it is possible that other treatments administered during this period also influence the genomic response. These

potential confounders remain difficult to resolve in the clinical setting. Observations of similar gene expression patterns in other patient groups receiving blood transfusion, as well as investigations in subjects receiving semi-elective blood transfusion who are not critically ill, together with *in vivo* and *in vitro* laboratory studies may help to clarify the specific immunological effects of allogeneic blood transfusion.

It is also possible that significant numbers of residual allogeneic leukocytes persist even in leukodepleted packed red cells and thereby confound the analysis of patient mRNA levels as a result of contamination. This could be of particular concern in the setting of massive transfusion. However, it has been previously demonstrated that leukodepleted packed red cells contain no detectable DNA and that the postmortem analysis of patients receiving massive transfusions failed to detect foreign DNA (Graham *et al.*, 2007).

A potential limitation of our findings is the use of gene expression data at the mRNA level as opposed to analysing protein expression. However, as has been previously discussed, our previous experience with protein analysis in the acute setting has been disappointing, in that the assays utilised lacked sensitivity and accuracy *in vivo* (O'Dwyer *et al.*, 2006; O'Dwyer *et al.*, 2008). It has been suggested that the lack of correlation between mRNA and protein levels can be explained by the technical limitations of the methods used to determine protein expression levels (Hack, 2004). Such limitations are particularly evident when studying genes expressed at relatively low levels, given that commercially available kits lack the required sensitivity. Importantly however, investigators have reproducibly found positive correlations between mRNA and protein expressed at low levels using isotope coded affinity tags to quantify protein expression (Fu *et al.*, 2007)

The transfusion of PRBCs stored for longer periods was originally thought to be associated with equivocal effects (Marik & Sibbald, 1993). However, there is now an increasing body of evidence associating the use of older PRBCs with harm. Transfusion of older blood has been associated with an excess of infectious complications and multiple organ

failure in trauma patients (Offner *et al.*, 2002; Zallen *et al.*, 1999) and following cardiac surgery, transfusion of PRBCs stored for more than 28 days has been associated with a 2.5 fold increase in pneumonic complications (Leal-Noval *et al.*, 2008; Vamvakas & Carven, 1999). Following colorectal cancer surgery transfusion of PRBCs stored beyond 20 days more than doubled the risk of postoperative infections (Mynster & Nielsen, 2000). In addition, the duration of storage of PRBCs has been independently associated with an increased risk of death in trauma patients (Weinberg *et al.*, 2008) and following cardiac surgery (Koch *et al.*, 2008). Interestingly, the mortality data suggests that older PRBCs exert a detrimental effect only when transfused in larger quantities with smaller volume transfusion of older blood having little effect on mortality (Weinberg *et al.*, 2008). However, these data are largely retrospective and consequently prone to potential bias. Most recently the results of two large randomised controlled trials prospectively assessing the impact of the age of blood transfused in sepsis (Lacroix *et al.*, 2015) and cardiac surgery (Steiner *et al.*, 2015) demonstrated equivalence between the two groups. However, a recent meta-analysis incorporating trauma and cardiac surgery patients still suggests that in these cohorts those receiving 'older' PRBCs may be more susceptible to nosocomial infections (Ng *et al.*, 2015).

Prolonged storage of PRBCs can induce 'storage lesions' the clinical consequences of which are unclear (Lelubre & Vincent, 2011). In particular, whether these lesions could be responsible for increasing susceptibility to nosocomial infections and increasing mortality remains uncertain. Already in this Chapter we have described an association between the transfusion of PRBCs and an excess of infectious complications following major trauma. Importantly, here, we also offer a plausible mechanism for the detrimental effect of PRBCs by describing a pattern of gene expression that was consistent with immunosuppression and was independently associated with transfusion. Here we demonstrate that the administration of older PRBCs exacerbates this transfusion induced immunosuppressed phenotype, thereby perhaps providing a mechanism for further increasing susceptibility to nosocomial infections.

A reduction in IL-12, IL-23 and RORγt gene expression in association with an increase in TGF-β gene expression is consistent with an immunosuppressed phenotype. IL-12 is a pro-inflammatory cytokine, produced primarily by dendritic cells and macrophages, that vigorously promotes the differentiation of naïve T cells into an IFN-γ producing T_h1 phenotype (Del Vecchio *et al.*, 2007). T_h1 cells are an essential link between innate and adaptive immunity and are particularly important for effective bactericidal activity on intracellular pathogens (Kitamura *et al.*, 2005). IL-23 is a cytokine, again produced primarily by dendritic cells and macrophages, that promotes differentiation to a T_h17 phenotype (Korn *et al.*, 2009). RORγt is a transcription factor specific to terminally differentiated T_h17 cells (Korn *et al.*, 2009). T_h17 cells are a branch of the adaptive immune system and appear to deal primarily with organisms inadequately subdued by T_h1 or T_h2 immunity and that seem to require a very robust inflammatory response (Korn *et al.*, 2009). TGF-β is produced by multiple lineages of leukocytes and stromal cell (Li *et al.*, 2006). Although a paracrine source of TGF-β may be necessary for T_h17 development (Korn *et al.*, 2009) this cytokine is better known for inducing widespread apoptosis and limiting the pro-inflammatory T_h1 response (Li *et al.*, 2006).

Whilst we previously reported in this Chapter that expression of the prototypical anti-inflammatory gene, IL-10, increases in response to trauma and in response to allogeneic blood transfusion in trauma patients, in this cohort IL-10 gene expression was not associated with the age of the transfused PRBCs. It is plausible that this analysis was underpowered to detect such an association. We did however detect greater increases in another potent anti-inflammatory gene, TGF-β, in those patients receiving older PRBCs.

An association was also detected between decreasing FOXP3 and GATA-3 and the age of the PRBCs, transcription factors specific to the immunosuppressive T_{reg} cell response and the T_h2 response respectively (Vignali *et al.*, 2008; Zheng & Flavell, 1997) and as such these findings would not support the hypothesis that transfusion of older PRBCs induces a greater immunosuppressive response. However, an association was also detected between these

variables at baseline and the age of PRBCs prior to transfusion. Therefore the validity of the association at 24 hours is questionable. Furthermore, the multivariate analysis suggested that patient age was a more important variable in explaining gene expression variation in GATA-3 and FOXP3 than the median age of the PRBCs. Whilst this may also be true for the change in IL-23 gene expression over 24 hours an additional marker of reduced T_h17 activity, RORγt, remained independently associated with the transfusion of older PRBCs.

Although we hypothesise that the described gene expression profiles may potentially lead to an increased risk of subsequent infectious complications this analysis was not powered to detect such an association. These data do, however, provide a plausible mechanistic link between older PRBCs reducing the effectiveness of essential bactericidal functions and thereby rendering patients more susceptible to later infectious insults. This is an important distinction from suggesting that older PRBCs may directly introduce infection to the host. Earlier in this Chapter we have reported a median time lag of 7 days between the appearance of immunosuppressive gene expression patterns and the detection of a bacteraemia, which supports the concept that these changes are associated with an enhanced susceptibility to later infectious insults as opposed to representing an early immune response to infection.

This facet of the Chapter has a number of limitations. As has been previously discussed retrospective observational cohort studies are prone to systematic biases, unknown confounders and the possibility of Type 1 errors, and must be viewed as hypothesis generating. In this aspect of the Chapter, while the expression of a number of inflammatory mediators are significantly associated with the age of blood transfused, the accompanying r^2 values that we present are low implying that age of blood might account for only a small proportion of the variation in the gene expression. However, there will be multiple influences present in trauma patients influencing gene expression and it would be surprising if any single factor, such as age of blood, had a dominant effect on the inflammatory response. Our data suggests that the age of blood has a small, yet significant effect on gene expression and could contribute to clinical

outcomes. Importantly, unlike many other factors influencing the inflammatory response to major trauma, it is potentially modifiable. As before it is possible that unknown confounding variables were omitted from our multivariate regression analysis despite the inclusion of variables descriptive of shock on admission and at 24 hours, the ISS, the volume of transfused PRBCs and a common adjustment model with the same confounders considered in each case. There is no evidence-based consensus in the literature as to what constitutes 'old or 'young' blood so as a consequence rather that dichotomising the age of blood into inexact groups, the age of blood was examined as a continuous (ordinal) variable in order to minimise bias. Again this is a limitation of a retrospective observational analysis rather than a randomised controlled trial. The inclusion of patients receiving both large and small volume transfusions may obscure important associations as it has been suggested that small volume transfusion of old blood does not adversely affect mortality (Weinberg *et al.*, 2008). In addition to this the variability between patients receiving large and small volumes of PRBCs may mean that differences between the calculated (median) age of the blood transfused in different patients may be underestimated. Patient numbers preclude meaningful sub-analyses. There were some baseline associations between FOXP3 and GATA-3 gene expression and the age of transfused PRBCs, which introduces bias in the interpretation of alterations in T_{reg} and T_h2 cell types at 24 hours.

3.6 Conclusions

A well-developed cytokine response to severe trauma is present within 2 hours of the injury. Excess and rising key anti-inflammatory cytokines coupled with unchanged or falling levels of key pro-inflammatory cytokines and associated transcription factors describes an anti-inflammatory direction to this response over the first three days following injury. The magnitude of this response at 24 hours is associated with excess infections and with excess mortality. The primarily immunosuppressive inflammatory response to polytrauma may be exacerbated by the transfusion of blood products and by the age of the blood transfused.

Furthermore, transfusion was associated with an increased susceptibility to nosocomial infections and an increased risk of death. Further clinical and *in vitro* investigations will be required to clarify mechanisms and causation.

Chapter Four: The Influence Of Major Elective Abdominal Surgery & Allogeneic Transfusion On T-Helper Cell Related Candidate Gene Expression

This Chapter contains one published work:

'Perioperative blood transfusion is associated with a gene transcription profile characteristic of immunosuppression: a prospective cohort study' Fragkou PC*, **Torrance HD***, Pearse RM, Ackland GL, Prowle JR, Owen HC, Hinds CJ and O'Dwyer MJ. *Critical Care*. 2014; 18:541 PMID: 25270110 (***Joint First Authors**)

4.1 Introduction

The host immune response to major physiological insults has previously been thought to encompass a distinct early pro-inflammatory phase, termed the systemic inflammatory response syndrome (SIRS) followed sequentially by an anti-inflammatory phase, termed the compensatory anti-inflammatory response syndrome (CARS), (Hotchkiss & Karl, 2003). This immunological response is further expanded upon in Chapter one of this thesis. In the perioperative literature evidence supporting this paradigm in patients undergoing major non-cardiac surgery is limited to small studies that profile a limited selection of important plasma cytokines (Jhanji *et al.*, 2010; Novotny *et al.*, 2012). A further limitation of these studies is the selection of candidate cytokines, such as interleukin-6 (IL-6), based on the relative abundance of target protein as opposed to their unambiguous and reproducible effects on host immunity (Diehl & Rincon, 2002). As has been alluded to in Chapter three, advances in qualitative real-time polymerase chain reaction (qRT-PCR) technology now permit a more sensitive and widespread assessment of gene expression *in vivo* by quantifying messenger RNA (mRNA) levels as a surrogate (O'Dwyer *et al.*, 2006). Furthermore, careful selection of key cytokines in conjunction with specific transcription factors allows inferences to be made as to the activity of specific inflammatory pathways (Pachot *et al.*, 2005). Previously our group has described immune alterations following thoracotomy (White *et al.*, 2011) and severe polytrauma (Chapter three), and their relation to infectious complications utilising this methodology (White *et al.*, 2011).

Recent data challenges the sequential SIRS / CARS paradigm, particularly in the setting of severe sepsis and blunt trauma (Hotchkiss *et al.*, 2013; Xiao *et al.*, 2011). A novel emphasis on an early and more dominant immunosuppressive phenotype associated with an enhanced susceptibility to develop infectious complications is emerging. Importantly, high-risk patients undergoing major non-cardiac surgery also display an enhanced susceptibility to

hospital acquired pneumonia (Pearse *et al.*, 2006), with preliminary reports suggesting the presence of a postoperative functional immunoparalysis (Veenhof *et al.*, 2011).

The immunological picture is also clouded by allogeneic transfusion. The immunomodulating qualities of blood transfusion have long been appreciated and have even been exploited to prevent renal allograft rejection in the era prior to the development of effective immunosuppressant drugs (Opelz & Terasaki, 1978). The unintended clinical consequences of immune modulation by allogeneic blood in the perioperative period include an increased susceptibility to infectious complications and cancer recurrence (Cata *et al.*, 2013; Jensen *et al.*, 1992). As inpatient non-cardiac surgery has recently been associated with much higher than anticipated mortality rates it is now imperative that all potentially avoidable causes of excess morbidity in this population are investigated and addressed (Pearse *et al.*, 2012). Whilst preoperative anaemia is associated with a doubling of in-hospital mortality it remains unclear whether the anaemia itself or its treatment with allogeneic blood is responsible (Baron *et al.*, 2014). Furthermore, perioperative blood transfusions are not universally associated with an increase in complications particularly when patients receiving leukodepleted blood are included (Cata *et al.*, 2013; Lapierre *et al.*, 1998). Also lacking from the current literature is an analysis of alterations in key inflammatory pathways associated with the transfusion of leukodepleted blood following major elective surgery.

In this chapter we explore the hypothesis that major gastrointestinal surgery is associated with an early postoperative immunosuppressive pattern of gene expression, which is quantitatively associated with susceptibility to later infectious complications and that this phenomenon may be compounded by allogeneic transfusion.

4.2 Methods

The Methods for this Chapter are fully outlined in Chapter two of this thesis.

4.3 Results

4.3.1 Patients

119 patients (mean age 65, range 57-72, 62% male) undergoing scheduled major abdominal surgery were included. Patient characteristics are shown in Table 4.1.

4.3.2 Change In mRNA Levels Over The Initial 48 Hours

IL-10 mRNA levels increased from baseline to 24 hours (Figure 4.1A). FOXP3 and GATA-3 mRNA levels both decreased over this period ($p<0.0001$ for both)**.** IFN-γ (Figure 4.1B), IL-12, T-bet ($p<0.0001$ for all) and TNF-α ($p=0.002$) mRNA levels all decreased over the initial 24 hours. TNF-α mRNA levels then increased from 24 hours to 48 hours ($p=0.0002$,) but the median 48 hour TNFα mRNA level remained less than the baseline level ($p=0.004$). IL-23 ($p=0.002$), IL-27 ($p=0.01$) and RORγt ($p<0.0001$) mRNA levels all decreased over the initial 24 hours.

The TNF-α / IL-10 mRNA ratio decreased over the initial 24 hours (Figure 4.1C). The TNF-α / IL-10 mRNA ratio then increased from 24 to 48 hours ($p<0.0001$) but the 48 hour median value remained below the median baseline value ($p<0.0001$). With the exception of TNF-α, no changes in gene expression were detected between 24 and 48 hours for any of the other mediators assessed. Quantification of the median fold-change in mRNA levels from baseline to 24 hours is presented in Supplementary Table 10.

4.3.3 Postoperative Infections

44 (37 %) patients developed postoperative infections a median of 9 (IQR 5 – 11) days following the operation. The sites of infection and isolated organisms are shown in Table 4.2. Patients developing infections stayed longer in hospital (14 (8 – 19) vs 7 (5 – 10) days, $p<0.0001$). A range of demographic and clinical data did not distinguish between those who did and did not develop infection (Table 4.1).

No associations were detected between the baseline mRNA levels and the occurrence of postoperative infectious complications. Levels of IL-10 mRNA at 24 hours (p=0.007, Figure 4.2A) and 48 hours (p=0.03) were higher in those who developed infectious complications.

Levels of FOXP3 (p=0.02) and GATA-3 (p=0.005) mRNA at 24 hours were lower in those who developed infectious complications.

Levels of T-bet (p=0.04), IL-12 (p=0.04) and TNF-α (p=0.03, Figure 4.2B) mRNA at 24 hours and levels of TNF-α mRNA at 48 hours (p=0.03) were lower in those who developed infectious complications.

Levels of IL-23 (p=0.02) and RORγt (p=0.04) mRNA at 24 hours were lower in those who developed postoperative infections. Changes in IL-27 mRNA levels were not associated with postoperative infectious complications.

The TNF-α / IL-10 mRNA ratio at 24 hours (p=0.004, Figure 4.2C) and at 48 hours (p=0.005, Figure 4.2D) was lower in those patients developing infectious complications.

4.3.4 In-Hospital Mortality

Three (2.5%) patients died in hospital on the 7th, 9th and 63rd postoperative day. None of the following, age, diabetes, smoking history, ASA, length of operation, cancer diagnosis, planned ICU admission, preoperative immunosuppression or postoperative infections was found to be associated with in-hospital mortality.

IL-23 mRNA levels at 24 and 48 hours (p=0.03 for both), RORγt mRNA levels at 48 hours (p=0.04) and the TNF-α / IL-10 mRNA ratio at 24 hours (p=0.01) were lower in those who died. IL-10 mRNA levels at 48 hours were higher in those who died (p=0.008).

Table 4.1 - Patient characteristics of those undergoing major abdominal surgery

	Infection, *n=44 (37%)*	**Infection free,** *n=75 (63%)*	***p*-value**
Age (years)	66 (59 – 75)	64 (56-71)	*Ns*
Male sex	27 (61%)	47 (63%)	*Ns*
Diabetes	8 (18%)	12 (16%)	*Ns*
Current smokers	10 (23%)	14 (19%)	*Ns*
Smoking history	21 (48%)	43 (57%)	*Ns*
Cancer diagnosis	24 (55%)	53 (71%)	*Ns*
Preoperative immunosuppression	6 (14%)	11 (14%)	*Ns*
Duration of operation (mins)	243 (176 – 313)	195 (142 – 295)	*Ns*
Endoscopic surgery	8 (18%)	24 (32%)	*Ns*
Planned postoperative intensive care unit admission	34 (77%)	50 (66%)	*Ns*
ASA grade 3 or 4	13 (30%)	23 (31%)	*Ns*
By surgical specialty n (%)			
General surgery	4 (44%)	5 (55%)	
Upper gastrointestinal	9 (33%)	18 (67%)	
Colorectal	18 (37%)	31 (63%)	
HPB	11 (37%)	19 (63%)	
HPB + colorectal	1 (33%)	2 (67%)	
General surgery + colorectal	1 (100%)	0 (0%)	*Ns*[1]
Intraoperative blood transfusion (%)	6 (14%)	4 (5%)	*Ns*
Blood transfusion first 24hr (%)	10 (23%)	5 (7%)	0.02
In hospital death	1 (2%)	2 (2.5%)	*Ns*

Data are described as medians with interquartile range or numbers with percentages in parenthesis. *ASA*, American Society of Anaesthesiologists. *HPB*, Hepato-Pancreato-Biliary. [1] represents a Fisher's exact test incorporating all surgical specialties. *Ns,* non-significant.

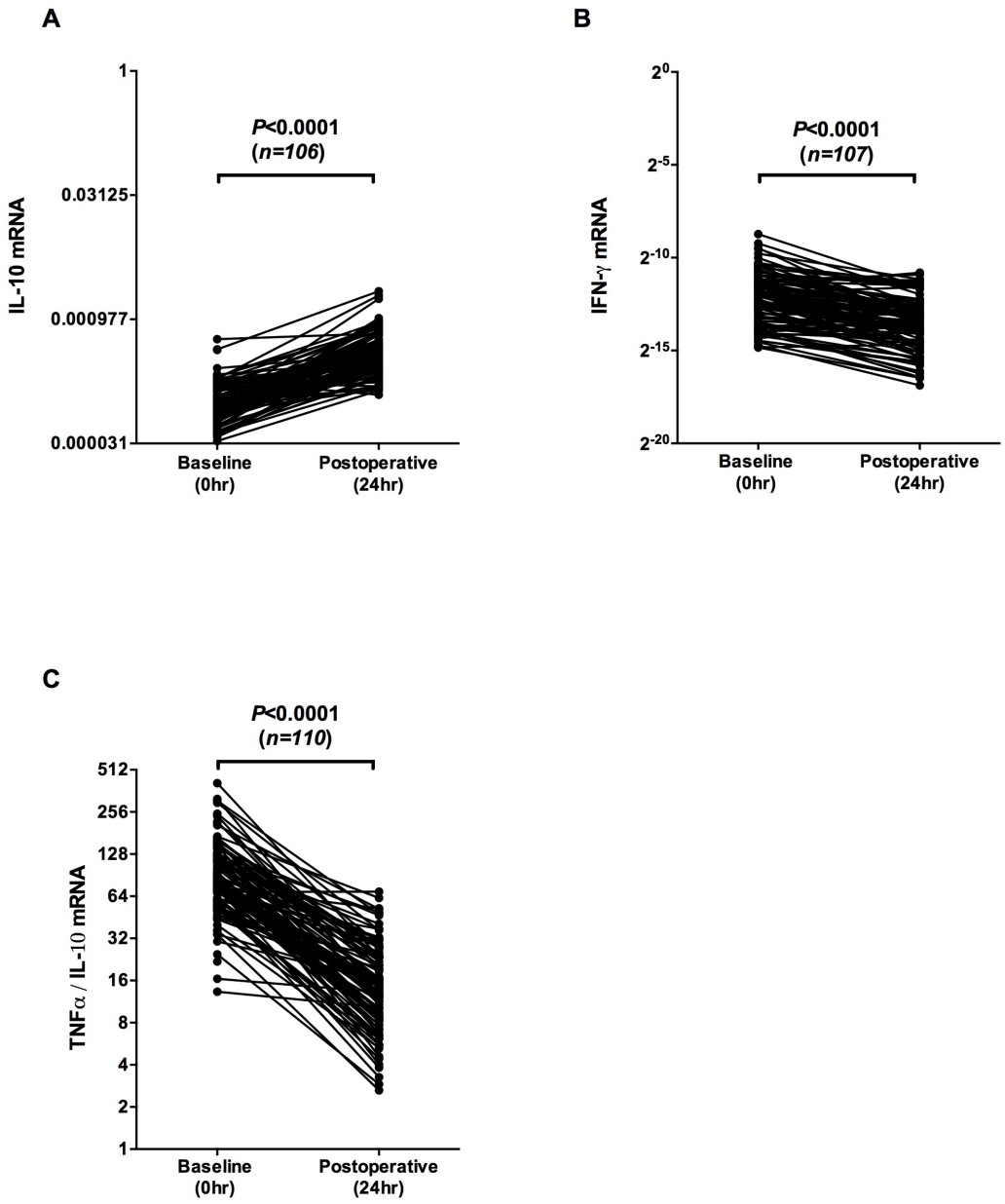


Figure 4.1 - Perioperative changes in gene expression

Graphs represent the change in mRNA levels from the preoperative sample (baseline) to 24 hours postoperatively. The Y axis is scaled to Log_2, mRNA results are expressed as a relative quantification ratio between candidate and the reference genes.

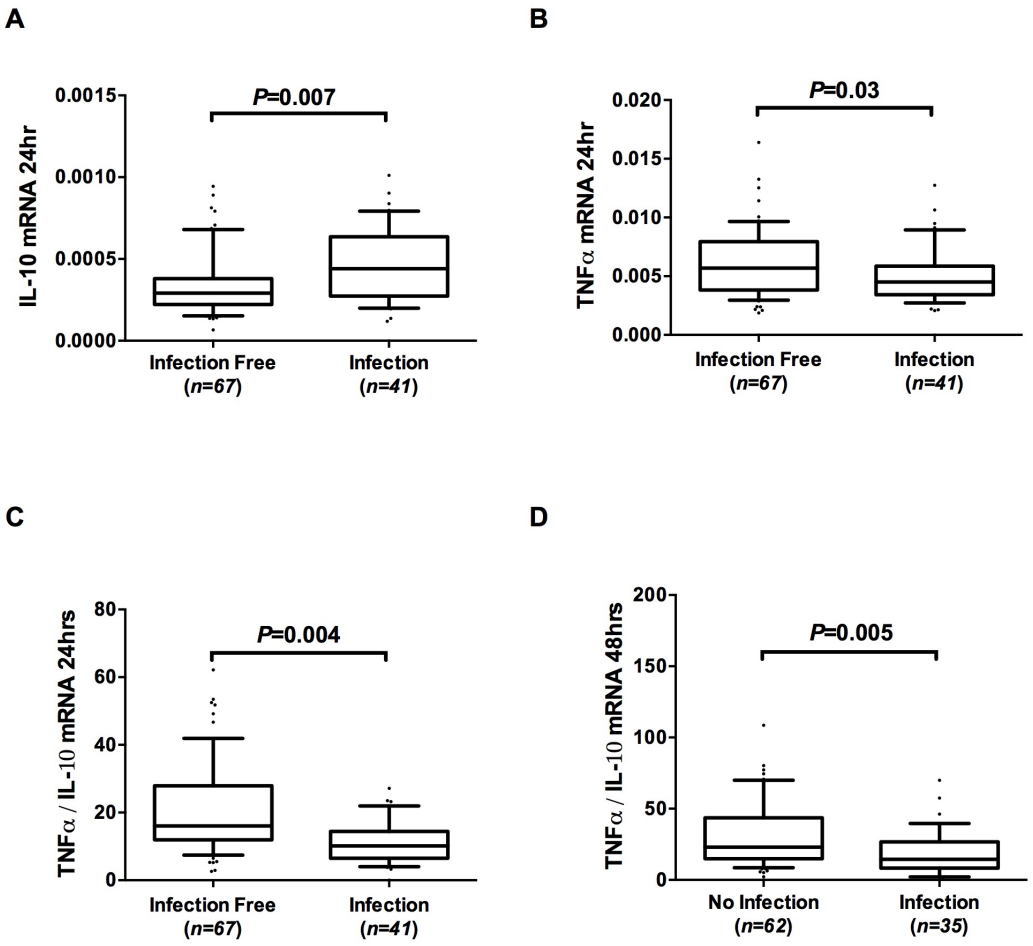


Figure 4.2 - Gene expression & postoperative infection

Graphs A-B demonstrate univariate analysis of candidate gene mRNA levels at 24 hours and the occurrence of nosocomial infectious complications. Graph C demonstrates a univariate analysis of a ratio of candidate gene mRNA levels and the occurrence of nosocomial infectious complications. Graph D demonstrates the relationship with candidate genes assessed at 48 hours. Graphs A-D represent median, interquartile range (*box*) and the 10th and 90th percentile (*whiskers*). All results are expressed as a relative quantification ratio between candidate and the reference genes.

Table 4.2 - Outline of the infectious complications in the postoperative period following major abdominal surgery

Infection site	Number of episodes	Organisms	Median time to development of infection (days)
Bloodstream	11	*E. coli* (4) ESBL *E. coli* (1) *E. coli* + *E. faecium* (1) *E. faecium* (1) *K. pneumoniae* (1) *S. aureus* (1) *P. aeruginosa* (2)	17 (6 – 28)
Pneumonia	9	Culture negative (5) *Candida glabrata* (1) *K. pneumoniae* (1) Organism of the coliform group (1) *S. maltophilia* (1)	7 (4 -16)
Surgical Site	24	Culture negative (11) *B. fragilis* (1) *E. coli* (4), ESBL *E. coli* (1) Mixed anaerobes (1) Organism of the coliform group (2) Organism of the coliform group + *E. faecalis* (1) Organism of the coliform group + MRSA(1) Organism of the coliform group + *S. aureus* (1) *P. aeruginosa* (1)	10 (4 -14)
Urinary tract	15	*Candida albicans* (1) Culture negative (2) *E. coli* (6) ESBL *E. coli* (1) Mixed growth (2) Organism of the coliform group (2) *P. aeruginosa* (1)	8 (6 -12)
Intra-abdominal	17	Culture negative (6) *E. coli* (2) *E. coli* + *E. faecium* (1) ESBL *E. coli* + VRE(1) *E. faecalis* (1) *E. faecium* + *Candida albicans* (1) *Candida albicans* (1) *E. cloacae* (1) *E. cloacae* + Organism of the coliform group (1) *E. Aerogenes* + *P. aeruginosa* (1) VRE (1)	9 (7 -14)
Intravascular catheter-related	2	*K. pneumoniae* (1) Organism of the coliform group (1)	15 (3 -27)
Skin	1	Varicella zoster virus (VZV)	30

Data refer to the number of episodes of infection from a particular site. Some patients may have more than one episode of infection. The number in parenthesis in the organisms column the number of episodes of infection attributable to that organism. ESBL, Extended-spectrum beta-lactamase; MRSA, methicillin-resistant S. aureus; VRE, Vancomycin-resistant Enterococcus. Data are described as median and interquartile range

4.3.5 Blood Transfusion

A total of 15 (13%) patients were transfused a median of 2 units (IQR 1-2) of PRBC in the initial 24 hours. Ten of these patients received intraoperative transfusion, eight postoperative transfusion and three patients received PRBC both intra-operatively and postoperatively.

No other blood product was transfused during this time period. Older patients (p=0.0002) and those with a diagnosis of cancer (p=0.02) were more likely to receive PRBC transfusions. Smokers were less likely to receive a blood transfusion (p=0.04). The requirement for blood transfusion was not related to the duration of the operation, whether the procedure was endoscopic or open or the ASA grade (Table 4.3). Patients receiving PRBC were more likely to develop postoperative infections (OR 5.5 (1.3 – 12.8); p=0.02 (Fisher's exact test), Figure 4.3A) and were more likely to die in-hospital (OR 15.7 (1.3 – 185.3); p=0.04 (Fisher's exact test), Figure 4.3B).

4.3.6 Blood Transfusion & Gene Expression

On univariate analysis, IL-12 mRNA levels at 24 hours (p=0.02) and TNF-α mRNA levels at 48 hours (p=0.01) were lower in those who received a blood transfusion in the first 24 hours postoperatively. IL-23mRNA levels at 24 (p=0.007) and at 48 hours (p=0.03) and RORγt mRNA levels at 24 hours (p=0.004) and at 48 hours (p=0.006) were lower in those receiving a blood transfusion over the first 24 hours postoperatively. The TNF-α / IL-10 mRNA ratio at 24 hours (p=0.0006) and at 48 hours (p=0.01) was lower in patients receiving blood transfusion over this period (Figure 4.4A-H).

FOXP3 mRNA levels at 24 hours (p=0.004) and at 48 hours (p=0.02) and GATA-3 mRNA levels at 24 hours (p=0.02) were lower in those who received blood transfusion in the initial 24 hours postoperatively. IL-10, IFN-γ, T-bet and IL-27 mRNA levels were unrelated to blood transfusion. The number of units of blood transfused was unrelated to gene expression.

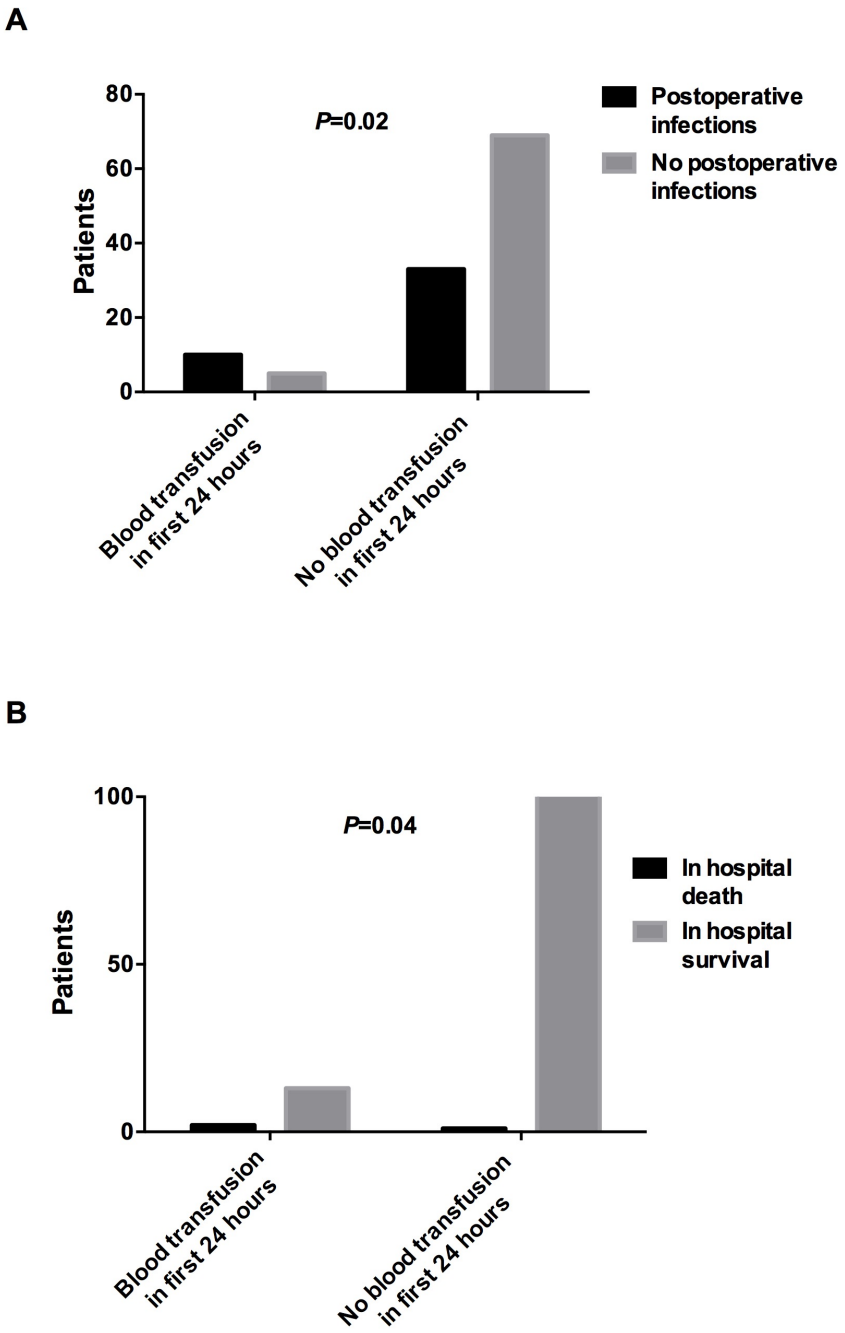


Figure 4.3 - Perioperative blood transfusion, infectious complication & survival

Graph A represents the proportions of patients developing postoperative infectious complications amongst those patients receiving a blood transfusion in the first 24 hours and in those patients not receiving a blood transfusion during this time period. Graph B represents the proportions of patients either dying in hospital or surviving to hospital discharge amongst those patients that received a blood transfusion in the first 24 hours (2 patients died out of a total of 15) and in those patients that did not receive a blood transfusion during this time period (1 patient died out of a total of 104).

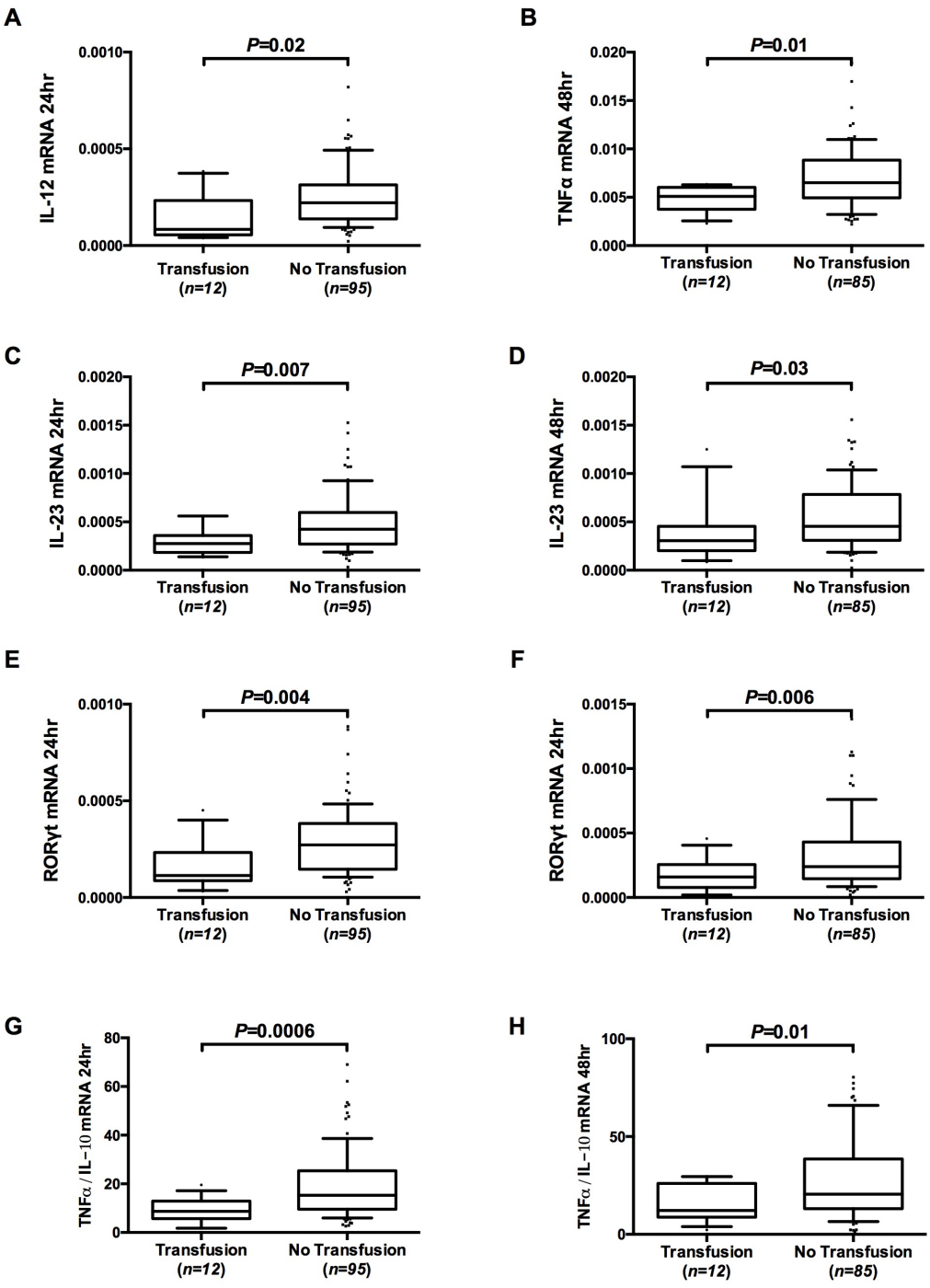


Figure 4.4 - Perioperative blood transfusion & gene expression

Graphs A-H represent the mRNA levels of candidate genes following scheduled abdominal surgery in those patients who received a transfusion prior to this time point and in those patients that did not. (median, interquartile range (*box*) and the 10th and 90th percentile (*whiskers*)). All results are expressed as a relative quantification ratio between candidate and reference genes.

4.3.7 Mean Age Of The Transfused Blood & Gene Expression

The age of the blood was calculated as the number of days from collection from a blood donor to transfusion. No association was detected between the mean age of the units of blood transfused and gene expression for any of the selected genes.

4.3.8 Postoperative Infections

With the exception of perioperative blood transfusion, a range of demographic and clinical data did not adequately distinguish between those who did and did not develop infection (Table 4.1).

4.3.9 Multivariable Analysis

Selection of variables to be included in the multivariate analysis is described in the methods section of this Chapter. IL-12, RORγt, TNF-α / IL-10 and FOXP3 mRNA levels at 24 hours along with RORγt, TNF-α / IL-10 and FOXP3 mRNA levels at 48 hours were independently associated with transfusion in the first 24 hours (Supplementary Table 11). There was no independent association between GATA-3 and IL-23 mRNA levels at 24 hours and between IL-23 and TNF-α mRNA levels at 48 hours and blood transfusion within the first 24 hours.

There was an independent association between the occurrence of infectious complications and the requirement for blood transfusion in the first 24 hours postoperatively (Supplementary Table 12).

Table 4.3 - Characteristics of patients requiring perioperative blood transfusion following scheduled abdominal surgery

	Transfused n=15 *(13%)*	**Not transfused n=103** *(87%)*	***p*-value**
Age (years)	77 (72 – 81)	63 (56-70)	0.0002
Male sex	9 (60%)	64 (62%)	*Ns*
Diabetes	3 (20%)	18 (17%)	*Ns*
Current smokers	0 (0%)	24 (23%)	0.04
Smoking history	4 (27%)	60 (58%)	0.02
Cancer diagnosis	14 (93%)	62 (60%)	0.02
Preoperative immunosuppression	4 (29%)	12 (12%)	*Ns*
Duration of operation (mins)	240 (150 – 400)	212 (145 – 296)	*Ns*
Endoscopic surgery	2 (13%)	30 (29%)	*Ns*
Planned postop ICU admission	13 (87%)	70 (68%)	*Ns*
ASA grade 3 or 4	9 (60%)	29 (28%)	*Ns*
By surgical specialty n (%)			
General surgery	0 (0%)	8 (7%)	
Upper gastrointestinal	4 (27%)	23 (22%)	
Colorectal	5 (33%)	44 (43%)	
HPB	5 (33%)	25 (24%)	
HPB + colorectal	1 (7%)	2 (2%)	
General surgery + colorectal	0 (0%)	1 (1%)	*Ns*[1]
Postoperative infections n (%)	10 (66%)	34 (33%)	0.02
In hospital death n (%)	2 (13%)	1 (1%)	0.04

Data are described as medians with interquartile range or numbers with percentages in parenthesis. mRNA levels are expressed as a relative quantification ratio between the candidate and the reference genes. HPB, Hepato-Pancreato-Biliary. [1] represents a Fisher's exact test incorporating all surgical specialties. *Ns,* non-significant.

4.4 Discussion

In this chapter we determined levels of mRNA for a panel of interlinked cytokines and related transcription factors and demonstrated that a specific pattern of altered gene expression is apparent within 24 hours of major gastrointestinal surgery. The four-fold up-regulation in expression of the archetypal anti-inflammatory gene, IL-10, in conjunction with decreasing gene expression for mediators descriptive of pro-inflammatory activity in the T_h1 and T_h17 pathways and a seven-fold decrease in the TNF-α / IL-10 gene expression ratio, points to the immunosuppressive nature of this response (Supplementary Table 10). A further analysis was carried out examining the effects of allogeneic transfusion following major gastrointestinal surgery, which displayed a pattern of gene expression consistent with greater immunosuppression when compared with a cohort not receiving a blood transfusion. This observation was independent of variables descriptive of the extent of surgical trauma. The gene expression data presented suggests that blood transfusion in this setting may be associated with specific immune defects in innate immunity and in T_h1 and T_h17 pathways. These patients also had an excess of postoperative infectious complications when compared with patients who did not receive a blood transfusion; an observation that was again independent of the duration of the surgical procedure.

The data we report are consistent with the recent reappraisal of the sequential SIRS / CARS paradigm in other settings (Hotchkiss *et al.*, 2013; Xiao *et al.*, 2011). A growing appreciation of the importance of an exaggerated immunosuppressive response has led directly to preliminary trials of immune stimulants in an effort to reduce the incidence of nosocomial infections in susceptible sepsis and trauma patients (Meisel *et al.*, 2009; Nakos *et al.*, 2002). Perioperative patients are a similarly susceptible group with comparable rates of nosocomial infections (Canet & Gallart, 2013). Indeed, there is precedent for immune stimulation in the perioperative period; subcutaneous IFN-γ facilitates release of pro-inflammatory bactericidal mediators, but does not influence infection or mortality rates (Schinkel *et al.*, 2001). However,

in order to demonstrate meaningful impacts on important postoperative endpoints trials must be adequately powered and utilise immunodiagnostics to target high-risk patients in conjunction with monitoring the treatment response.

IL-10 induces host immunosuppression through a variety of mechanisms, including inhibition of MHC class II expression on macrophages and suppression of pro-inflammatory cytokines and chemokines in a number of different cell types (Couper *et al.*, 2008). The cellular source of the excess IL-10 mRNA in our patients is unclear from the whole blood analysis performed. Although natural T_{reg} (nT_{reg}) cells have consistently been associated with the development of impaired immunity, levels of the nT_{reg} cell specific transcription factor FOXP3 have been shown to decrease over the postoperative period (Couper *et al.*, 2008) as was the case in this Chapter. Alternative sources of IL-10 unrelated to FOXP3 expression are abundant amongst innate and adaptive cell types and include inducible T_{reg} cells, T_h2 cells, dendritic cells, macrophages, mast cells, eosinophils, neutrophils, $CD4^+$ and $CD8^+$ T cells, B cells as well as damaged muscle or the endothelium (Ouyang *et al.*, 2011). Flow cytometry experiments would assist in identifying the source of IL-10.

Excess IL-10 enhances susceptibility to usually benign infectious pathogens (Meghari *et al.*, 2008). IL-10 gene expression can also be up-regulated directly by a pathogen as an evolutionary mechanism to evade host defenses (Carey *et al.*, 2012). We report that raised IL-10 gene expression on the first postoperative day increases the risk of infections diagnosed approximately a week later. It remains unclear whether this represents unrecognised infection present on day one or merely an immune environment conducive to later bacterial invasion. Whilst ultrasensitve microbial detection tests, such as 16s PCR, may confirm or refute the presence of circulating microbes in the early postoperative period it would be unsurprising to detect episodes of bacteraemia following gastrointestinal surgery and their relevance would therefore be uncertain. Whether suppressed pro-inflammatory pathways are a direct downstream effect of the excess IL-10 is not clear from these data but this hypothesis is

supported by animal models and *ex vivo* human data (Carey *et al.*, 2012). Furthermore, the immune defect is likely to be multifaceted with a possible additive effect resulting from pro-inflammatory pathways in conjunction with excess IL-10 gene expression. Whilst reduced gene expression of mediators descriptive of the pro-inflammatory T_h1 and T_h17 pathways are associated with infectious complications the TNF-α / IL-10 mRNA ratio separates these groups more clearly.

Multiple factors are likely to trigger and modulate the immune response in this setting. Both circulating endotoxin from the gastrointestinal lumen and the presence of endogenous intracellular 'alarmins' released following tissue trauma have been implicated (Marshall, 2010; Zhang *et al.*, 2010). Volatile anaesthetic vapours, hypnotics and analgesics are all likely to exert some influence on the immune phenotype observed in the perioperative period (Fahlenkamp *et al.*, 2014). The influence of these treatments are described in more detail in Chapter one of this thesis.

Although numerous small studies have suggested that perioperative allogenic blood transfusion may induce immune modulation which creates an environment that promotes both microbial growth and tumorigenesis their conclusions are limited by small cohort size, *in vitro* and *ex vivo* assays and insensitive protein assays (Blajchman, 2002; Decker *et al.*, 1996; Jensen *et al.*, 1992; Kirkley *et al.*, 1998; Nielsen *et al.*, 1991; van Twuyver *et al.*, 1991; Waymack *et al.*, 1986). These studies largely predate the routine leukodepletion of transfused blood and suggest a causal relationship between immune modulation and the infusion of allogeneic leukocytes; a biological effect which could be ameliorated by pre-storage leukodepletion (Blajchman, 2002). It cannot therefore be inferred that a similar immunological response will be observed following exposure to a leukodepleted product. In contrast, the packed red cells transfused in the study reported here were universally leukodepleted. The results we present suggest that the immunosuppression observed following perioperative transfusion may not be solely dependent on the presence of allogeneic leukocytes in the transfused blood. However,

despite a 3 log reduction in leukocyte numbers achieved following current filtration techniques some leukocytes do persist and these retain the ability to influence host immune responses (Blajchman, 2002).

In Chapter three, using a similar methodology we described an excess in blood stream infectious complications amongst severely injured trauma patients receiving leukodepleted blood transfusion in conjunction with a gene expression profile characterised by specific deficits in T_h1 and T_h17 pathways. Clearly patients who receive a blood transfusion outside of the context of a randomised controlled trial will differ from those who do not require transfusion and it is important to attempt to control for these factors. In the previously reported trauma cohort (Chapter three) the described observations were independent of the severity of injury and degree of shock at presentation. Similarly, in this perioperative cohort, although on univariate analysis there was a trend towards longer and therefore potentially more complicated surgery being associated with postoperative infections and to a lesser degree with blood transfusion, a multivariable analysis confirmed that the association between blood transfusion and both infectious complications and gene expression patterns were independent of the duration of surgery. Although we describe an association between blood transfusion, an immunosuppressive pattern of gene expression and excess infectious complications it may be that, despite adjustment for known confounders, the need for blood transfusion is a surrogate marker of severity of surgical stress or an unmeasured pre-morbid condition and thus a causative role in postoperative immune phenotype is suggested but not proven. However, the consistency of these data from two distinct patient populations, post major gastro-intestinal surgery patients and trauma patients, strengthens our underlying hypothesis.

The association between blood transfusion and infectious complications in the perioperative period has been well described previously (Bennett-Guerrero *et al.*, 2007; Horvath *et al.*, 2013; Jensen *et al.*, 1992). This association appears to be consistent amongst cohorts that received either whole blood or leukodepleted blood. The magnitude of the effect

size is potentially quite substantial with one recent estimate quantifying the increased risk of infection at 29% for each unit of blood transfused (Horvath *et al.*, 2013). That we were able to describe a similar association between blood transfusion and nosocomial infection in a smaller cohort of patients may be related to this effect size, in addition to the selection of an at-risk population using patient age, gastrointestinal surgery under general anaesthesia and the requirement for inpatient treatment as inclusion criteria. The postoperative infection rates we report are consistent with similar patient populations (Pearse *et al.*, 2014). This study also described an association between blood transfusion and mortality. However, whilst the mortality rate is consistent with similar populations (Pearse *et al.*, 2014), with only 3 deaths occurring in the cohort as a whole no conclusions regarding causation should be inferred from this association.

It is unsurprsing that we did not observe an association between increasing age of the transfused blood and either perioperative infectious complications or patterns of gene expression as this study was underpowered to detect such an association. There is much current debate surrounding the potential pathogenicity associated with blood stored for excessive periods prior to transfusion. In the perioperative period blood stored for longer periods has been linked to an excess of infectious complications (Leal-Noval *et al.*, 2003; Mynster & Nielsen, 2000; Vamvakas & Carven, 1999). .

A limitation of this Chapter is that the candidate gene approach necessarily excludes many genes with potent pro and anti-inflamatory effects and therefore provides an incomplete picture of the whole genome response. Measures utilised in this Chapter such as TNF-α / IL-10 gene expression ratios can give a broad indication of immune competence but could also be criticised for being too reductionist. It seems unlikely, however, that an exuberant pro-inflammatory response could occur in conjunction with a significant reduction in the TNF-α / IL-10 gene expression ratio and marked downregulation of T_h1 and T_h17 pathways. The candidate gene approach also allows us to exclude from our analysis cytokines with co-existent

pro and anti-inflammatory effects, such as IL-6, where the overall effect on patient phenotype remains unclear (Diehl & Rincon, 2002).

4.5 Conclusions

Major gastrointestinal surgery is associated with a distinctive gene expression pattern that includes a dramatic up-regulation of the anti-inflammatory cytokine IL-10, in conjunction with evidence of depressed pro-inflammatory immune pathways. The extent of this response is associated with an excess of infectious complications. The transfusion of leukodepleted allogeneic blood during or immediately after major gastrointestinal surgery is associated with patterns of gene expression consistent with immunosuppression and specific deficiencies in innate immunity and T_h1 and T_h17 pro-inflammatory immune pathways. A mechanistic link between allogenic perioperative blood transfusion, defects in essential bactericidal pathways and an excess of nosocomial infections is suggested but not proven. Further research is necessary in order to prove causation.

Chapter Five: MHC Class II Antigen Density On *In Vivo* Monocytes & Cultured Healthy Control *In Vitro* Monocytes, Strategies For *In Vitro* Reversal Of Immunoparalysis

This Chapter contains two published works:

'Features of postoperative immune suppression are reversible with interferon gamma and independent of IL-6 pathways' Longbottom ER*, **Torrance HD***, Owen HC, Fragkou PC, Pearse RM, Hinds CJ, O'Dwyer MJ. *Annals of Surgery.* (ePub Oct 2015) PMID: 26445474 (***Joint First Authors**)

'Systemic Inflammatory Response Syndrome After Major Abdominal Surgery Predicted by Early Upregulation of TLR4 and TLR5' Lahiri R, Derwa Y, Bashir Z, Giles E, **Torrance HD**, Owen HC, O'Dwyer MJ, O'Brien A, Stagg AJ, Bhattacharya S, Foster GR, Alazawi W. *Annals of Surgery.* (ePub May 2015) PMID: 26020106

ELISA data on the Hepato-Pancreato-Biliary surgical sub-cohort of patients was reported in this paper.

5.1 Introduction

Following major surgery and severe polytrauma patients experience a period of immune suppression, which can predispose to the development of postoperative infections (Mokart *et al.*, 2011). Infectious complications result in considerable morbidity and are observed in over 30% of older patients undergoing major surgery (Pearse *et al.*, 2014) and up to 70% of patients suffering severe polytrauma (Chapter three). Postoperative immune dysfunction is mediated partly by tissue injury and the consequent release of intracellular alarmins such as mitochondrial DNA fragments (Zhang *et al.*, 2010). The cellular and transcriptomic features of tissue damage induced immune modulation are now well characterised (Gaudilliere *et al.*, 2014; Pillay *et al.*, 2012; Xiao *et al.*, 2011). Key reproducible cellular features of this response include a consistent fall in monocyte HLA-DR (mHLA-DR) expression (Cheron *et al.*, 2010; Mokart *et al.*, 2011; Wakefield *et al.*, 1993) and an expansion of cells with phenotypic similarities to myeloid derived suppressor cells (Gaudilliere *et al.*, 2014; Pillay *et al.*, 2012; Pillay *et al.*, 2013). These alterations are quantitatively associated with infectious complications and poor functional recovery (Cheron *et al.*, 2010; Gaudilliere *et al.*, 2014; Mokart *et al.*, 2011). Although the immune suppressive effect of major tissue damage has been shown to be reversible with interferon gamma (IFN-γ) treatment in the context of traumatic injuries (Dries *et al.*, 1994; Nakos *et al.*, 2002; Polk *et al.*, 1992), in the perioperative period the specific additional immunomodulating effects attributable to such factors as prolonged anaesthetic administration, opioid analgesics, premorbid conditions and blood transfusion require further consideration (Matsuoka *et al.*, 2001; O'Dwyer *et al.*, 2015; Vallejo *et al.*, 2004).

There is similar consistency amongst the transcriptomic data available following major surgery and severe polytrauma (Baigrie *et al.*, 1992; Mokart *et al.*, 2005a; Xiao *et al.*, 2011). A consistent feature in the literature is the elevation of IL-6 and IL-10 levels in patients with

features of functional immune suppression and the correlation between circulating levels of this cytokine and infectious complications.

Although many essential pro-inflammatory effects are mediated through IL-6 pathways this cytokine has also been shown to drive differentiation of T helper cells to a T helper cell type 2 (T_h2) subtype and to inhibit T_h1 maturation through a Suppressor of Cytokine Signalling 1 (SOCS1) dependent suppression of IFN-γ signalling (Diehl & Rincon, 2002; Sofi *et al.*, 2009). These latter effects could be broadly construed as anti-inflammatory and may induce an immune environment in which host bactericidal abilities are impaired, thereby increasing susceptibility to infectious complications. Furthermore, the purported mechanism of action suggests that restoring IFN-γ activity may reverse the downstream consequences.

IL-10 on the other hand is a prototypical anti-inflammatory cytokine, originally described as a product of T_h2 cells, acting to inhibit T_h1 cytokine production (Fiorentino *et al.*, 1989). Latterly production of IL-10 has been described from a wide variety of cell types including monocytes, macrophages, dendritic cells as well as other T cell subsets (Couper *et al.*, 2008). Previous research has demonstrated that IL-10 mediated STAT-3 phosphorylation (Liu *et al.*, 2013b) and E3 ubiquitin-ligase March-I activation (Mittal *et al.*, 2015; Mittal & Roche, 2015) can impede antigen presentation. In addition to this IL-10 is known to activate the PI3K-Akt-GSK signalling pathway, thus suppressing inflammatory gene expression (Antoniv & Ivashkiv, 2011).

GM-CSF is a growth factor that has been utilised in the sepsis literature as a global immune stimulant (Bo *et al.*, 2011; Mathias *et al.*, 2015). Treatment has been shown to improve the survival, proliferation, phagocytosis, and the bactericidal ability of neutrophils and monocytes (Meisel *et al.*, 2009). In addition to this it has been shown to upregulate monocyte MHC class II molecule expression as well as increasing endotoxin-induced *ex vivo* pro-inflammatory cytokine production (Flohe *et al.*, 2003).

In this Chapter we hypothesise that increased levels of IL-6 and IL-10 following major surgery may promote an immunosuppressed phenotype that can be reversed with IFN-γ or GM-CSF. In order to explore this hypothesis we aimed to describe the patterns of IL-6 and IL-10 production in response to major gastrointestinal surgery and the relationship between both cytokines and infectious complications. We also aimed to explore the role of IL-6 and IL-10 dependent pathways in postoperative immune suppression and to determine whether this immune suppression might be reversed by IFN-γ or GM-CSF.

5.2 Methods

The Methods for this Chapter are fully outlined in Chapter two of this thesis.

5.3 Results

5.3.1 Patients

5.3.1.1 VISION

108 patients (mean age 65, range 46–87, 59% male) undergoing elective major abdominal surgery were included. 41 (38%) patients developed a postoperative infection. Further patient characteristics have previously been outlined in Chapter four (Tables 4.1 & 4.2).

5.3.1.2 METS

A comparable cohort to the VISION study made up of 12 patients (median age 68, IQR 65-78, 42% male), undergoing elective major abdominal surgery were recruited.

5.3.2 Postoperative Infections

In the perioperative cohort infectious complications developed a median of 9 (IQR 5–11) days following the procedure with those patients developing infections staying longer in hospital (14 (9–19) vs. 7 (5-9) days, $p<0.0001$). A range of demographic and clinical data did not distinguish between those who did and did not develop infection.

5.3.3 Perioperative IL-6 & IL-10 levels

IL-6 and IL-10 levels increased 19-fold and 5-fold respectively from baseline to 24 hours postoperatively ($p<0.0001$). These levels were unchanged between 24 and 48 hours postoperatively for IL-6 (Figure 5.1A & B), but fell for IL-10 ($p=0.0004$). Baseline IL-6 and IL-10 levels were higher in patients with a diagnosis of cancer, $p=0.02$ and $p<0.05$, Figure 5.1C & D). None of age, sex, smoking history, immunosuppression or diabetes was associated with baseline levels of either cytokine. At 24 hours greater levels of IL-6 and IL-10 were assayed in patients who would subsequently develop infections ($p<0.0001$ and $p=0.0019$, respectively; Figures 5.2A & B). This association was observed with both cytokines in intra-abdominal infections ($p=0.004$ and $p=0.0019$, respectively; Figures 5.2C & D) and surgical site infections ($p=0.001$ and $p=0.0009$, respectively; Figures 5.2E & F) but not pneumonia. IL-6 and IL-10 levels at 24 hours were directly correlated with the length of the procedure (Spearman's ρ 0.26, $p=0.01$ and Spearman's ρ 0.23, $p=0.03$, respectively). The levels were lower in patients who underwent endoscopic procedures ($p=0.0001$ and p=0.0007, respectively) and were higher in patients who received an epidural, in conjunction with their general anaesthetic ($p=0.006$ and $p=0.03$, respectively).

A multivariable linear regression model confirmed that the association between IL-6 or IL-10 levels at 24 hours and postoperative infectious complications remained when corrected for the differences in the above variables.

This pattern was replicated at 48 hours however with only IL-6 levels showing associations with those patients subsequently developing infections ($p=0.003$) and higher in patients who had longer procedures (Spearman's ρ 0.23, $p=0.03$). Both IL-6 and IL-10 levels were lower in patients who had endoscopic procedures ($p=0.0002$ and $p=0.0076$, respectively) and higher in patients who received an epidural ($p=0.02$ and $p<0.05$, respectively). The association between IL-6 levels at 48 hours and infectious complications again remained when corrected for these variables.

Table 5.1 - Assayed IL-6 & IL-10 serum levels & their relationship to late nosocomial infection following major abdominal surgery

	Infection *n=41 (40%)*	**Infection free** *n=67 (60%)*	***p*-value**
IL-6: VISION			
Pre-Op	3.7 (2.8 – 13)	3.0 (2.4 – 4.8)	*Ns*
24hrs	101 (49 – 242)	39 (19 – 92)	0.0002
48hrs	80 (31 – 170)	34 (16 – 79)	0.003
IL-10: VISION			
Pre-Op	0.8 (0.4 – 1.5)	0.8 (0.4 – 1.3)	*Ns*
24hrs	5.2 (3.5 – 8.2)	2.8 (2.1 – 4.7)	0.0019
48hrs	2.5 (1.7 – 4.9)	2.1 (1.5 – 4.2)	*Ns*

Data are described as medians with interquartile range. Recorded as pictograms / mL. Ns, non-significant.

Table 5.2 - Assayed IL-6 & IL-10 concentrations in the METS cohort

	Concentration pg / mL
IL-6: METS	
Pre-Op	2.8 (1.1 – 4.0)
24hrs	228 (75 – 470)
48hrs	137 (55 – 168)
IL-10: METS	
Pre-Op	0.4 (0.2 – 1)
24hrs	3.9 (1.5 – 6.8)
48hrs	1.7 (1.1 – 2.5)

Data are described as medians with interquartile range. Recorded as (pg) pictograms / mL

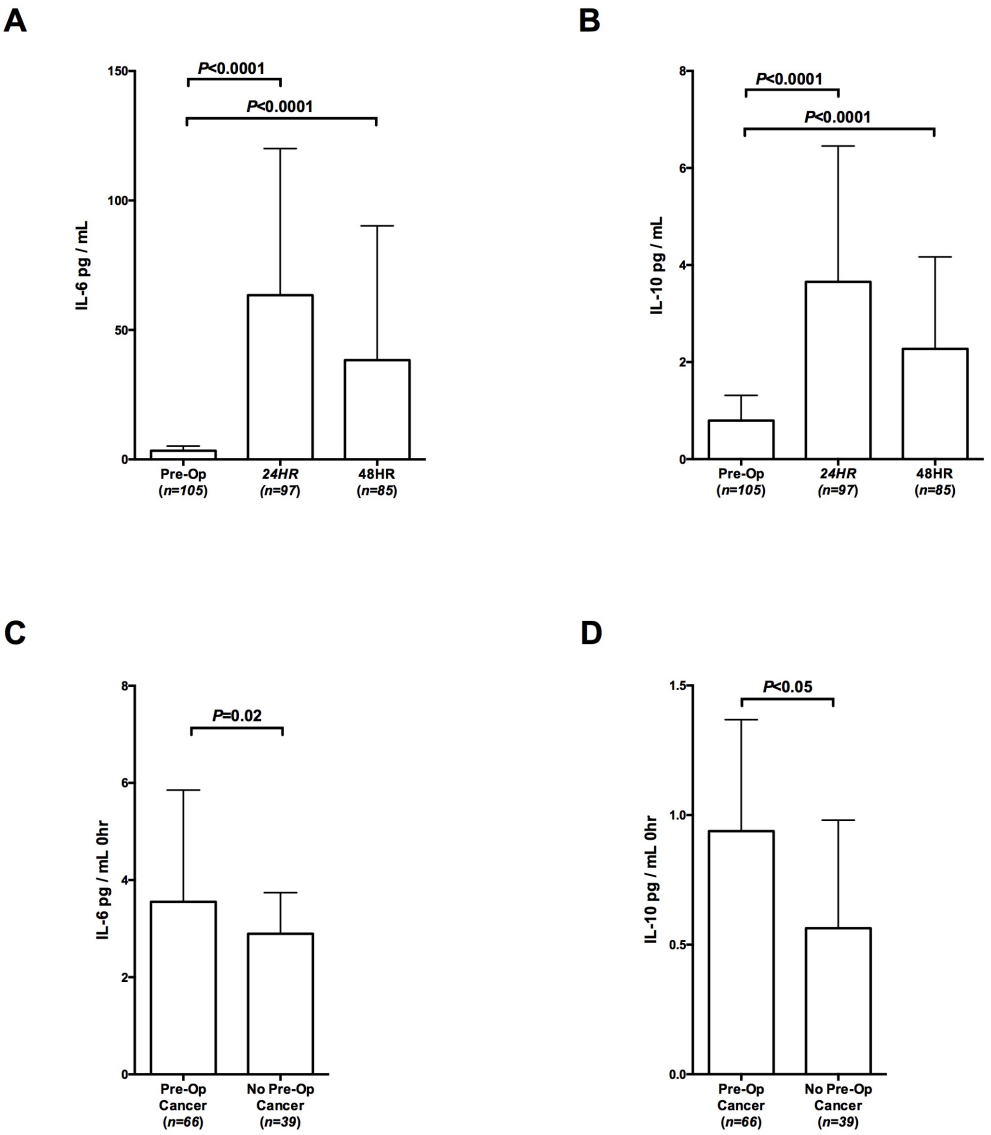


Figure 5.1 - IL-6 & IL-10 plasma levels, the influence of surgery & perioperative cancer

Changes in IL-6 (A) and IL-10 (B) concentration following major gastrointestinal surgery. Preoperative IL-6 (C) and IL-10 (B) protein levels were raised in patients who had a diagnosis of cancer. All data displayed as median and interquartile range; pg indicates picograms.

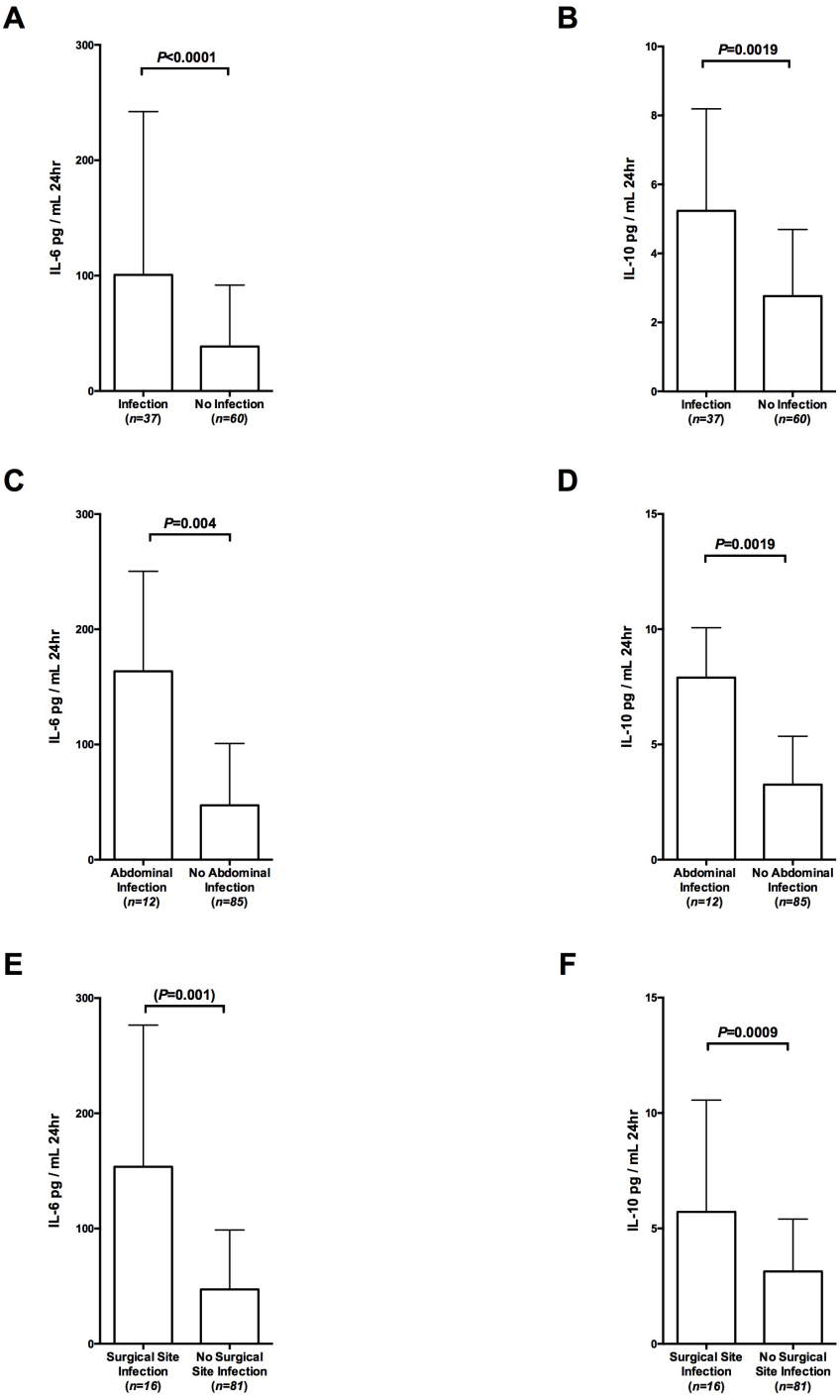


Figure 5.2 - Differences in IL-6 & IL-10 levels between those patients suffering any postoperative infection

All postoperative Infections (A & B). abdominal infection (C & D) or postoperative surgical site infection (E & F). Infections were defined according to the CDC criteria. All data displayed as median and interquartile range; pg indicates picograms.

The METS cohort demonstrated comparable temporal changes in IL-6 and Il-10 levels (Table 5.2), however as a smaller cohort (n=12) they were not powered to detect clinical correlations.

5.3.4 Effect Of Elective Major Abdominal Surgery On Monocyte HLA-DR Expression

A total of 12 consecutive patients from the METS study were recruited with $CD14^{+}$HLA-DR analysis performed on a flow cytometer at 3 time points; preoperatively, at 24 hours and 48 hours. At 24 and 48 hours there was a significant reduction in the number of mAb/C of HLA-DR (p<0.0001) when compared to the preoperative sample.

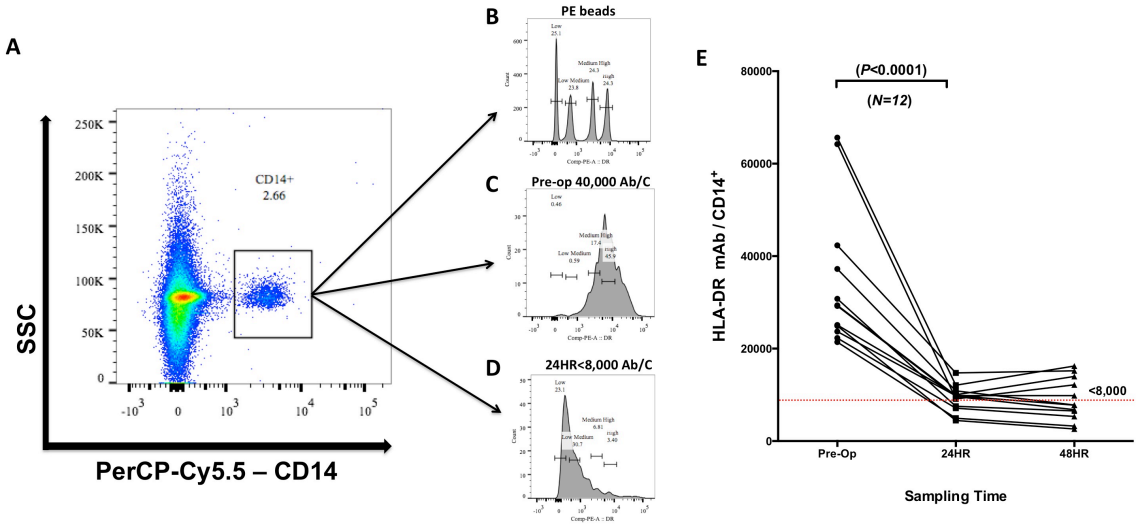


Figure 5.3 - Effect of elective major abdominal surgery on monocyte HLA-DR expression

Representative flow cytometry plots (A-D): EDTA anticoagulated blood was immediately stained with the combination CD14 / HLA-DR antibody, cells were gated on CD14 (A). PE coated beads were used to calibrate the experiment, these gave four defined population; low, low-medium, medium-high and high to allow accurate gate placement (B). A histogram plot showing preoperative (C) and postoperative (D) HLA-DR expression on the gated $CD14^{+}$ cells. Using geometric mean fluorescent intensity (*g*MFI) linear regression was performed to calculate the number of antibodies per $CD14^{+}$ cell (mAB / $CD14^{+}$). This showed a statistically significant change from baseline, graph displays all patients (*n=12*), with the red dotted line representing a level of 8,000 mAb/C (E).

5.3.5 Effect Of Postoperative Serum On Healthy Control mHLA-DR Expression & Following IL-6 Neutralisation

Serum was collected from 8 patients preoperatively and again at 24 hours. Details of these patients are given in Supplementary Tables 13 & 14. Healthy control mHLA-DR geometric MFI fell when incubated with serum collected 24 hours following major surgery when compared with *g*MFI levels following incubation with preoperative serum (Figure 5.3A, *P*=0.008, n=8). Pre-incubation with IL-6 neutralising antibody did not alter this reduction in geometric MFI (Figure 5.3B, *P*=0.95). Serum from a further 8 patients was collected preoperatively and at 24 hours. Patient characteristics outlined in Supplementary Table 13.

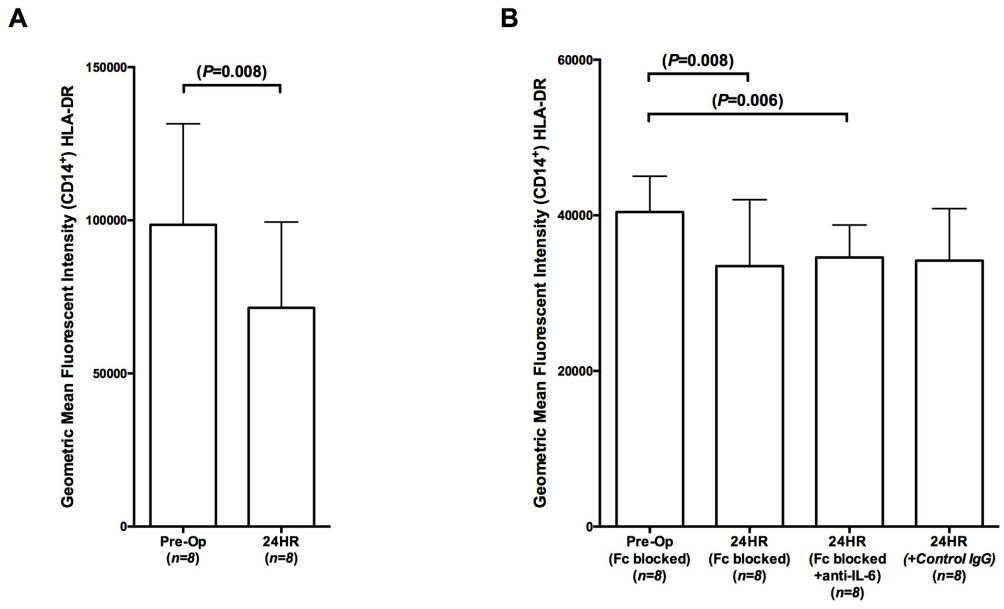


Figure 5.4 - Perioperative serum, healthy PBMCs, & the effects of IL-6 neutralisation

Postoperative serum decreases the level of mHLA-DR on cultured PBMCs obtained from healthy controls (A). The addition of an Fc blocker to the culture medium was necessary to eliminate nonspecific antibody actions (B). All data (A & B) from 2 independent experiments. All data displayed as median and interquartile range, HLA-DR quantified as geometric MFI on $CD14^+$ cells. Patient characteristics outlined in Supplementary Table 13.

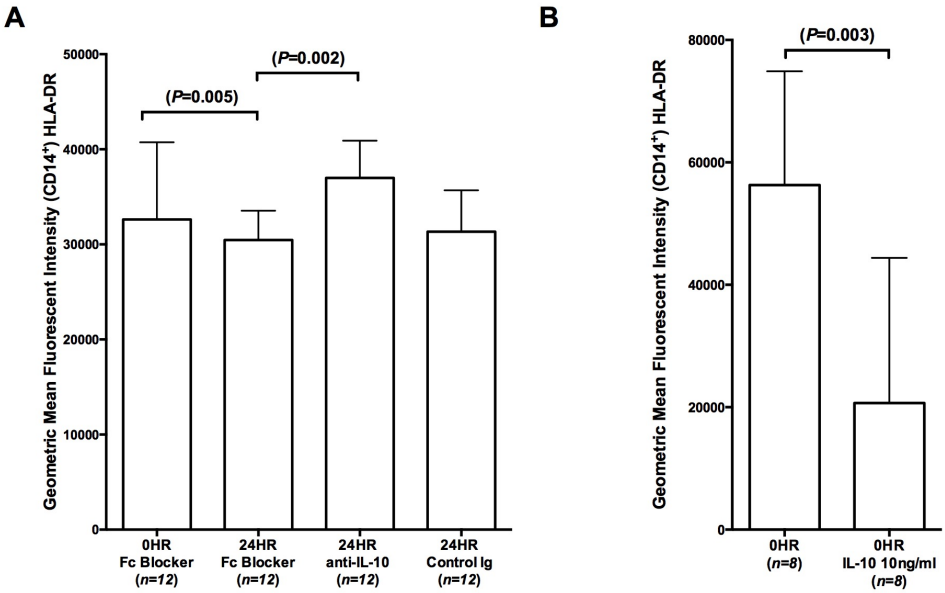


Figure 5.5 - Effects of postoperative serum on healthy control monocyte HLA-DR expression following IL-10 neutralisation or recombinant IL-10 incubation

Postoperative serum decreases the level of mHLA-DR on cultured Fc blocked PBMCs obtained from healthy controls, this decrease was reversed with the pre-incubation in IL-10 blocking antibody (A). The addition of an Fc blocker to the culture medium was necessary to eliminate nonspecific antibody actions. Incubation of recombinant IL-10 with preoperative serum decreased the level of mHLA-DR PBMCs (B). (A) Data from 3 independent experiments. (B) Data from 2 independent experiments. All data displayed as median and interquartile range, HLA-DR quantified as geometric MFI on $CD14^+$ cells. Patient characteristics outlined in Supplementary Tables 14 & 15, respectively.

5.3.6 Effect Of Postoperative Serum On Healthy Control mHLA-DR Expression & Following IL-10 Neutralisation & Recombinant IL-10 Incubation

Serum was collected from 12 patients preoperatively and again at 24 hours. Healthy control monocyte HLA-DR *g*MFI fell when incubated with serum collected 24 hours following major surgery when compared with *g*MFI levels following incubation with preoperative serum (Figure 5.4A, *P*=0.005, n=12). Pre-incubation with IL-10 neutralising antibody increased the mHLA-DR geometric MFI to preoperative values (Figure 5.4A, *p*=0.002).

Serum from a further 8 patients was collected preoperatively and incubated with recombinant IL-10, this caused the mHLA-DR geometric MFI to fall (Figure 5.4B, *p*=0.003) in

a similar manner to the postoperative serum. The patient characteristics for both experiments are outlined in Supplementary Tables 14 & 15, respectively.

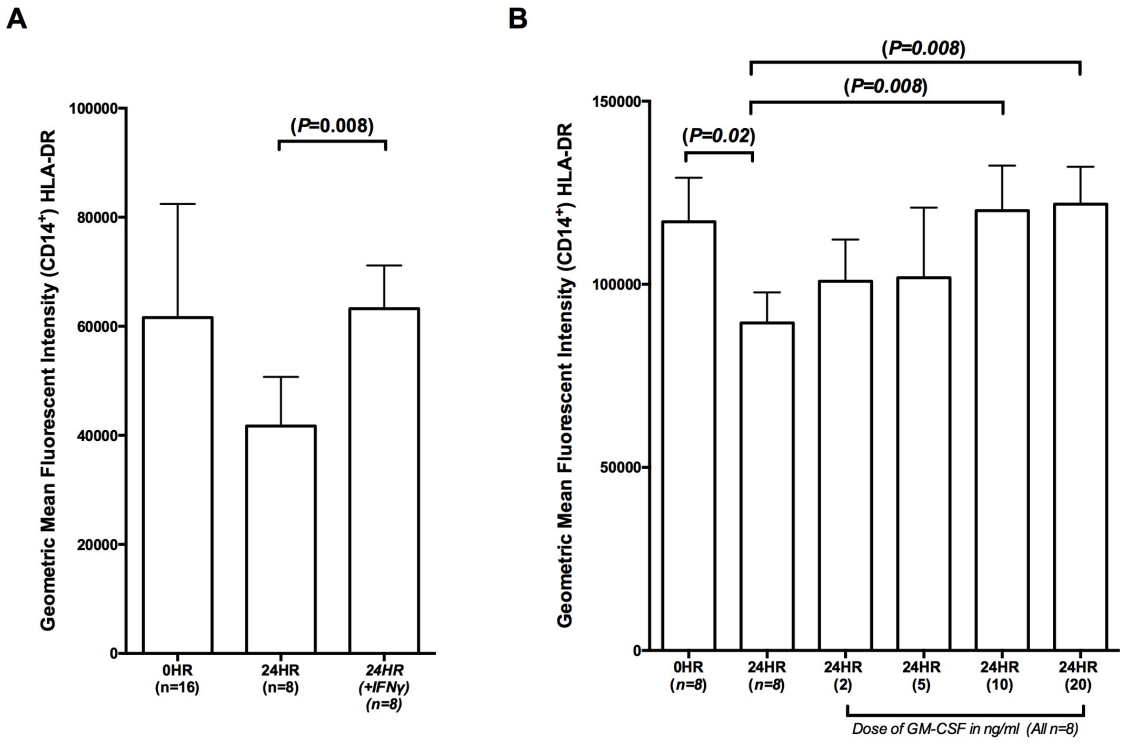


Figure 5.6 - Effects of postoperative serum on healthy control monocyte HLA-DR expression following IFN-γ or GM-CSF stimulation

Postoperative serum decreases the level of mHLA-DR on cultured PBMCs obtained from healthy controls comparison (A & B). When postoperative serum was co-cultured with IFN-γ (250 IU), mHLA-DR levels were restored to those levels seen with preoperative serum (A). The same is seen with increasing doses of GM-CSF (2 - 20ng/mL) (B). All data (A & B) from 2 independent experiments. All data displayed as median and interquartile range, HLA-DR quantified as geometric MFI on $CD14^+$ cells. Patient characteristics outlined in Supplementary Tables 16 & 17, respectively.

5.3.7 Effect of Postoperative Serum On Healthy Control mHLA-DR Expression Following IFN-γ & GM-CSF Stimulation

Again there was a fall in mHLA-DR gMFI following incubation with serum taken at 24 hours (Figures 5.5 A & B). Co-incubation with IFN-γ resulted in an increase in mHLA-DR gMFI (p=0.008, figure 5.5A, n=8). This restoration to preoperative levels was also seen with

co-incubation with GM-CSF in a dose dependent manner (p=0.008, figure 5.5B, n=8). The patient characteristics for both experiments are outlined in Supplementary Tables 16 & 17, respectively.

These data were also re-analysed excluding those patients with preoperative immunosuppression. A similar fall in mHLA-DR *g*MFI following incubation with serum taken at 24 hours was demonstrated which was prevented by co-incubation with IFN-γ (n=7) or GM-CSF (n=7).

5.4 Discussion

In this Chapter we have determined plasma levels of IL-6 and IL-10 protein in patients over 45 years of age undergoing elective surgery involving the gastrointestinal tract and described an independent association between higher postoperative levels of both cytokines and later nosocomial infections. We have shown that at 24 and 48 hours postoperatively there is a marked reduction in the density of HLA-DR receptors on patient monocytes. In addition to this we have also demonstrated that serum collected in the postoperative period contains soluble mediators, which reduce the antigen presenting capabilities of healthy monocytes as measured by HLA-DR expression. Our data suggests that IL-10, but not IL-6, dependent pathways are one of the essential mediators of this immune suppressed phenotype. Treatment with IFN-γ and GM-CSF *in vitro* reverses the induced deficit in the antigen presenting capabilities of monocytes exposed to postoperative serum.

The association between IL-6 and IL-10 levels and postoperative infections is not unexpected and has been previously described (Klava *et al.*, 1997; Mokart *et al.*, 2002; Mokart *et al.*, 2005b). Unsurprisingly, we also report that levels of both cytokines are higher in those patients undergoing lengthier procedures. These procedures are likely to be more complex with a higher probability of postoperative complications. Importantly, however, we have found that the association between the levels of both cytokines and postoperative infections is independent

of the duration of the surgical procedure. It is also well recognised that postoperative patients display features of immune suppression (Mokart *et al.*, 2011; Wakefield *et al.*, 1993). However, whereas previous investigators have reported alterations in the properties of specific cell subtypes or their cell surface markers (Gaudilliere *et al.*, 2014; Wakefield *et al.*, 1993) we demonstrate that immune suppression is mediated, at least in part, by soluble compounds that remain 24 hours following surgery, as opposed to the direct effect of either tissue damage, anaesthesia or endotoxin release on immune cell subtypes. These data may be of crucial importance in developing potential treatments to alleviate postoperative immune suppression.

The key link between the tissue damage characteristic of major surgery or severe polytrauma and the subsequent inflammatory response is the release of alarmins (Oppenheim & Yang, 2005). Alarmins are a group of structurally diverse compounds, which include high-mobility-group box (HMGB) proteins and mitochondrial DNA (mtDNA), which are released following tissue damage as cells undergo physiological stress or necrosis (Chan *et al.*, 2012). These are then recognised by a wide variety of pattern recognition receptors (PRRs), which include the membrane bound Toll-like receptors (TLRs) and the cytoplasmic NOD-like receptors (NLRs) (Chan *et al.*, 2012). Activation of PRRs induces an enzymatic cascade, which results in down-stream phosphorylation of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which in turn alters cytokine transcription.

IL-6 is a cytokine that is consistently upregulated acutely following activation of this cascade (Xiao *et al.*, 2011). Furthermore, following traumatic injury elevated levels of IL-6 are seen concurrently with evidence of immune suppression and reduced antigen presentation capabilities (Gouel-Cheron *et al.*, 2012). This association, together with the consistent observation that higher IL-6 levels and infectious complications are linked following major tissue damage raises the possibility that in this scenario IL-6 functions not in its traditional pro-inflammatory role but rather to promote an immune suppressed phenotype. This hypothesis is supported by evidence from animal models and *in vitro* data describing the anti-inflammatory

effects of IL-6 acting through a classical signalling pathway involving the membrane bound IL-6 receptor and SOCS1 and STAT3 dependent mechanisms which ultimately inhibit IFN-γ production (Diehl & Rincon, 2002; Scheller *et al.*, 2011; Sofi *et al.*, 2009). Although IL-6 antagonists such as tocilizumab are in clinical use, it is imperative that prior to considering their potential use following major surgery evidence is available that the increased IL-6 observed in this scenario is of significance as a specific mediator of poor clinical outcomes as opposed to being a biomarker of a poor clinical outcome (Smolen *et al.*, 2013). In the case of postoperative immune suppression our data suggests that although elevated IL-6 levels are associated with clinical correlates of immune suppression such as postoperative infectious complications it does not play a key role in impairing the antigen presenting capabilities in the postoperative period. Interestingly, it has recently been reported that the development of postoperative inflammatory complications and infection in particular, is preceded by increased expression of TLR4 and TLR5 on an intermediate ($CD14^{++}CD16^{+}$) monocyte population (Lahiri *et al.*, 2015). These same patients displayed higher resting and TLR-stimulated IL-6 levels in the early postoperative period in conjunction with enhanced activity in the NF-κB signalling pathway (Lahiri *et al.*, 2015).

IL-10 plays a pivotal regulatory role in the immune system, classically providing a regulatory role, preventing the development of chronic pro-inflammatory states (Saraiva & O'Garra, 2010). However in both trauma and major surgery this cytokine is consistently upregulated early post-insult (Mokart *et al.*, 2002). These elevations may be induced by circulating DAMPS or PAMPS, stimulating IL-10 production either via a TLR (specifically TLRs 2, 4 and 9) or TLR-independent pathways in macrophages and myeloid dendritic cells (Ng *et al.*, 2013). Following TLR stimulation, activation of adaptor molecules (myeloid differentiation primary-response protein 88 (MYD88) and TIR-domain-containing adaptor protein inducing IFN-β (TRIF)) occurs (Boonstra *et al.*, 2006) causing triggering of extracellular signal-regulated kinases (ERK1 and 2), p38 and NF-κB downstream signalling. The activation of ERK is key to IL-10 production, with ERK deficient cells (Agrawal *et al.*,

2006) or ERK inhibitors (Kaiser *et al.*, 2009) affecting IL-10 production. In the *in vitro* model, circulating DAMPS will certainly exist in patient serum and may stimulate IL-10 production from healthy monocytes further potentiating the immunosuppressive effects of the IL-10 already contained in the serum.

IFN-γ also plays an important regulatory role in IL-10 production via ERK- and p38-dependent signalling; with further downstream signalling leading to reduced TLR-induced IL-10 production via inhibition of the IL-10 promoter (Hu *et al.*, 2006). This pathway may be one of the secondary mechanisms observed *in vitro*.

In this cohort elevated IL-10 levels are associated with clinical correlates of immune suppression such as postoperative infectious complications as well as a reduction in mHLA-DR antigen density. A previous study in a septic cohort of patients described HLA-DR molecules being re-endocytosed and retained intracellularly in monocytes, with their data suggesting that this phenomenon is partially mediated by IL-10 (Fumeaux & Pugin, 2002). This phenomenon may partly explain our findings as IL-10 is known either directly (and indirectly via antigen presentation cells) to inhibit T-cell responses (Mittal & Roche, 2015) thus increasing patients' susceptibility to postoperative nosocomial infection.

Our results indicate that both IFN-γ and GM-CSF independently restore HLA-DR expression in affected monocytes. There are conflicting reports in the surgical literature on the effect of postoperative IFN-γ administration. In patients with colonic cancer IFN-γ appears to increase HLA-DR expression on PBMCs (Wiesenfeld *et al.*, 1995) whereas in other patients undergoing gastrointestinal surgery IFN-γ administered as part of *in vitro* experiments failed to reconstitute defective pro-inflammatory cytokine production (Wiesenfeld *et al.*, 1995). In the literature there is limited evidence of GM-CSF administration to perioperative patient cohorts, however there is an increasing evidence basis for targeted GM-CSF treatment in sepsis with a number of feasibility studies demonstrating raised mHLA-DR antigen density as well as

increases in pro-inflammatory cytokine productions, reductions in ventilator days and ICU stay (Mathias *et al.*, 2015; Meisel *et al.*, 2009).

Our data demonstrates that the *in vitro* administration of IFN-γ and GM-CSF can reverse features of defective antigen presenting capacity following major surgery in patients both with and without cancer. Our use of a well-validated, standardised biomarker of immune suppression (mHLA-DR) of particular relevance in the prediction of septic complications enhances the potential clinical applicability of these results (Docke *et al.*, 2005; Gouel-Cheron *et al.*, 2012; Meisel *et al.*, 2009). Further mechanistic work is required to identify the downstream pathways mediated.

There are a number of inherent strengths to this Chapter. We have studied a clinically relevant at-risk population selected by using age over 45 years old as an inclusion criterion. This ensures that the event rate of our primary outcome, infectious complications following elective surgery, is sufficiently high at 38% and is in keeping with recent data from similar cohorts (Pearse *et al.*, 2014). As all patients were undergoing elective surgery this allows each patient in the *in vitro* analysis to act as their own control. This is important as a significant number of these patients had a diagnosis of cancer or were immunocompromised and the use of healthy control serum as a comparator would be inappropriate. Finally, we describe a relatively novel method of identifying perioperative immune suppression by culturing healthy monocytes in postoperative serum in conjunction with the well-validated methodology of defining immune suppression by observing a decrease in mHLA-DR expression (Fumeaux & Pugin, 2002; Meisel *et al.*, 2009).

Limitations of this Chapter include; not assaying circulating IFN-γ protein levels in the postoperative period and the wide variability in the surgical procedures performed resulting in a heterogeneous patient population. We chose not to assay circulating IFN-γ protein levels due to the insensitivity of the IFN-γ assay in clinical conditions where in our experience IFN-γ levels are frequently undetectable.

5.5 Conclusions

IL-6 and IL-10 levels increase following major surgery and higher levels are associated with an increased susceptibility to postoperative infections. Serum obtained from postoperative patients induces an immunosuppressive response through IL-10 dependent but IL-6 independent pathways, which is reversible with IFN-γ or GM-CSF administration. Although, increased IL-6 levels may be useful as a biomarker of impending infectious complications our data do not support a causal relationship. Further studies will be required to define the mechanism action of IL-10.

Chapter Six: Conclusions Of This Thesis

6.1 The Novel Findings Of This Thesis

6.11 The Targeted T_h cell Transcriptomic Response To Trauma & Major Abdominal Surgery

Chapters three and four begin by describing the hyper-acute (<two hours) and acute (<24 hours) transcriptomic changes that take place following severe polytrauma and major abdominal surgery, respectively. Specifically, when comparing whole blood mRNA expression of targeted T_h cell cytokines and transcription factors from a cohort of healthy controls to those suffering severe polytrauma, it is apparent in the latter that there is a well-developed inflammatory response that is already present on admission. The hyper-acute elevation of the prototypical anti-inflammatory cytokine IL-10 implies that there is a strong immunosuppressive component to this. This is compounded by hyper-acute reductions in other classically pro-inflammatory T_h cell related cytokines and transcription factors, interestingly with the exception of a hyper-acute rise in IFN-γ mRNA. At 24 hours this immunosuppressive picture continues with the IL-10 mRNA levels peaking, and all other assayed cytokines and transcription factors falling below the levels assayed in the healthy control cohort. Although this upregulation of IL-10 with adaptive immune suppression has been demonstrated on analysis of the leukocyte transcriptome in the seminal 'Genomic Storm' paper (Xiao *et al.*, 2011). These data were however assayed at a less homogeneous and significantly a much later time point following injury (<12 hours), meaning that large numbers of the patients had received extensive resuscitation or emergency surgery prior to their initial blood sample. These data discussed in the thesis also reflect a more up to date resuscitation practice, which now minimises the use of use of clear fluids.

Interestingly, in the cohort of major surgical patients, postoperative whole blood mRNA sampling at 24 hours, using the same selection of cytokines and transcription factors, showed a similar rise in IL-10 with downregulation of T_h1 and T_h17 pathways. The similarities between these cohorts suggests that these patterns may be primarily induced by tissue damage

mediated DAMPS released into the circulation, as there was no evidence of shock or ischaemia-reperfusion injury in the major abdominal surgical cohort.

It is very significant that the magnitude of the early anti-inflammatory response or indeed the failure to mount or maintain a pro-inflammatory response was closely associated with the acquisition of subsequent infections in both cohorts, and with death in the trauma cohort. In both cohorts, patients suffering nosocomial infection had longer durations of hospital stay, with higher incidences of multi-organ failure in the trauma cohort.

6.12 The Influence Of Allogeneic Transfusion On The T_h Cell transcriptome In Trauma & Major Abdominal Surgery

The subsequent aspects of Chapters three and four then described the influence that allogeneic blood transfusion has on whole blood transcriptome. Allogeneic transfusion, a commonly administered treatment in both trauma and elective major abdominal surgery cohorts, has long been associated with immunomodulatory effects (Rohde *et al.*, 2014), (Figure 6.1). However there has been little descriptive *in vivo* evidence of how transfusion may modulate the immune system, particularly in severe polytrauma where patients often receive significant volumes of blood products. In the trauma cohort patients were dichotomised into two groups based on whether they received a transfusion or not, prior to their admission sample (immediate transfusion), or prior to their 24 hour sample (early transfusion). We found that a specific pattern of altered gene expression is apparent within two hours of the injury and that the immunosuppressive nature of this response is exacerbated by the transfusion of blood. A similar picture was seen at the 24 hour sampling point with allogeneic transfusion suppressing the pro-inflammatory gene expression profile while simultaneously increasing IL-10 gene expression. Although those patients transfused had an increased severity of injury and shock state, multivariate models, aimed to account for confounding variables, implied the independence of the gene expression changes. In addition to this there was an increased

incidence of nosocomial infection, specifically blood stream infections, and an increased risk of death in those patients transfused prior to their 24 hour blood sample.

The role of allogeneic transfusion was also investigated in the perioperative cohort, demonstrating that those patients receiving a perioperative blood transfusion following major gastrointestinal surgery displayed patterns of gene expression consistent with greater immunosuppression when compared with a cohort not receiving a blood transfusion (Figure 6.1). Again this observation was independent of variables descriptive of the extent of tissue injury. These patients also had an excess of postoperative infectious complications when compared with patients who did not receive a blood transfusion.

Following on from describing the influence of allogeneic transfusion, the final aspects of Chapter three investigated the effects of the duration of storage of PRBCs. There has been considerable interest in literature regarding the role that age related storage lesions of stored blood play in modulating the immune response. We identified that patterns of altered gene expression consistent with greater immunosuppression were associated with the transfusion of older PRBCs. These data suggested a reduced activity in the pro-inflammatory T_h1 and T_h17 pathways with reductions in the gene expression of the prototypical T_h1 polarising cytokine, IL-12, the T_h17 promoting cytokine, IL-23, and the T_h17 specific transcription factor RORγt.

In addition, with increasing age of transfused PRBCs there was also increased expression of the apoptotic and anti-inflammatory gene, TGF-β. This further points to the immunosuppressive nature of the response observed following the transfusion of older PRBCs, despite more recent RCTs in sepsis (Lacroix *et al.*, 2015) and cardiac surgery (Steiner *et al.*, 2015) pointing to equivalence between '*younger'* and '*older'* blood transfused. However a recent systematic review article (Ng *et al.*, 2015) incorporating trauma patients suggested that there may still be an increased risk of nosocomial infection with the transfusion of '*older'* blood. This may be due to the significantly larger volumes of PRBCs that this cohort of patients is transfused.

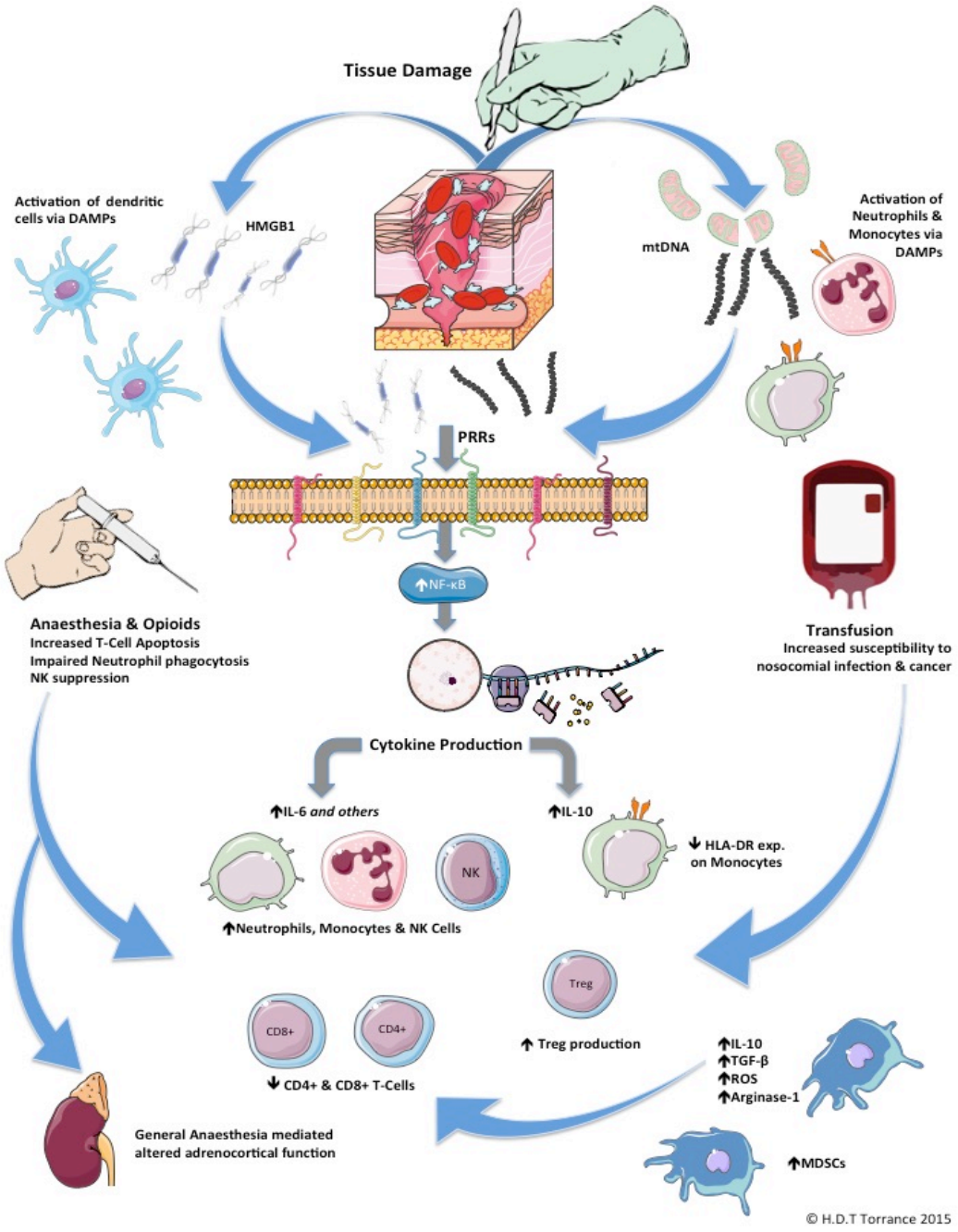


Figure 6.1 - Pathways of immune activation following tissue damage

Tissue damage leads to the release of Damage Activated Molecular Patterns (DAMPs) into the circulation, in this case illustrated by high-mobility-group box (HMGB) 1 and mitochondrial DNA (mtDNA), causing activation of pattern recognition receptors (PRRs). DAMPs also independently activate neutrophils, monocytes and dendritic cells. Activation of PRRs causes the triggering of signalling pathways and transcription factors such as NF-kB. NF-kB then translocates to the nucleus, promoting cytokine gene transcription. Protein translation results in the secretion of cytokines and chemokines.

Postoperatively, increases in neutrophils, monocytes and natural killer (NK) cells are seen along with later decreases in CD4+ and CD8+ T-cells. IL-6 and IL-10 consistently increase during this phase and IL-10 is associated with downregulation of HLA-DR expression on the surface of circulating monocytes. Immunosuppressive T_{reg} cells and myeloid derived suppressor cells (MDSCs) increase postoperatively. MDSCs cause T-cells suppression via the secretion of reactive oxygen species (ROS), arginine-1 as well as the immunosuppressive cytokines, IL-10 and TGF-β, amongst other mechanisms. Treg cells are known producers immunosuppressive cytokines; IL-10, TGF-β and IL-4.

The transfusion of blood and blood products is known to contribute to this immunosuppressive environment, while anaesthetics and opioids cause increase T-cell apoptosis, impair neutrophil phagocytosis and suppress NK cells suppression. Anaesthetics mediate secondary effects through altered adrenocortical function and central mechanisms.

6.13 HLA-DR Expression On *In Vivo* Monocytes & Cultured Healthy Control *In Vitro* Monocytes, Strategies For *In Vitro* Reversal Of Immunoparalysis.

In the final results Chapter (five) we determined plasma levels of IL-6 and IL-10 protein via ELISA in high-risk patients undergoing major abdominal surgery, describing an independent association between higher postoperative levels of both cytokines and later acquisition of nosocomial infection. Using a standardised international protocol (Docke *et al.*, 2005) we have shown that at 24 and 48 hours postoperatively there is a marked reduction in the density of HLA-DR receptors on patient monocytes. This widely published, internationally standardised, measure of immune competence validates the previous statements alluding to the immunosuppressive nature of the gene expression patterns in earlier Chapters.

Although the previous data described in this Chapter have been described in smaller cohorts, the functional aspects of these cytokines on healthy monocyte HLA-DR expression have not. Consequently we demonstrated that serum collected in the postoperative period contains soluble mediators, which reduce the antigen presenting capabilities of healthy monocytes. Our data suggests that IL-10, but not IL-6, dependent pathways are one of the essential mediators of this immune suppressed phenotype (Figure 6.1). Treatment with IFN-γ and GM-CSF *in vitro* reverses the induced deficit in the antigen presenting capabilities of monocytes exposed to postoperative serum. This opens up the possibility of these licenced immune-stimulants being used to treat perioperative patients in a targeted manner.

6.2 Strengths Of This Thesis

6.22 Trauma Research Infrastructure At The Royal London Hospital

One of the major factors that was central to the success of this thesis was the infrastructure that was already in place prior to my commencing this investigation. The Royal London Hospital is recognised as one of the UK leaders in terms of polytrauma research. The recruitment process that is in place, allowing the enrolment of patients within this hyper-acute

sampling window, has clear advantages in defining early immunological changes secondary to the tissue damage and shock, that was previously unavailable in the rest of the world. To facilitate this there is a research fellow rota, which operates from 08:00 to 20:00. This allows the systematic recruitment of all trauma patients eligible to be included in ACIT2. Although the ACIT2 recruitment model has now extended to national as well as international partners, this investigation utilised samples recruited solely from the RLH. As a consequence, all trauma patients were recruited within a single centre, with standardised pre-hospital care and resuscitation protocols. These protocols did not evolve during the recruitment phase discussed in this thesis.

The use of professional consent allows the systematic inclusion of all patients deemed eligible for recruitment by the trauma team leader. Great care is taken to ensure that patients or their LAR are approached as soon as appropriate after the acute phase of their admission. When professional consent has initially been given, only a small number of patients or relatives refuse participation when later approached for written consent, any that did were excluded from analysis. One of the most important qualities of the thesis is the strength of the clinical follow up conducted by the trauma research team. A daily ward round is performed to ensure that outcomes were collected prospectively; at that point patient consent or assents for samples obtained under professional consent were obtained and relevant temporal blood testing was performed.

A common criticism levelled at trauma research is survival bias; this is particularly relevant in coagulopathy research where patients receiving the 'optimal' ratios of PRBCs to FFP may not have been as severely injured as those who required large volumes of PRBCs to maintain an adequate MAP. As a result, in this cohort all patients were included in the analysis, despite their outcome, meaning that, despite the observational nature of the study design, survival bias was minimised.

6.23 Perioperative Research Infrastructure At The Royal London Hospital

Perioperative research at the Royal London Hospital is also internationally renowned, due to the work of Professor Rupert M Pearse. As a result it recruits patients not only for in-house-designed studies, which often have national and international arms, but it also acts as a UK site for large international studies conceived outside the group. The wide diversity of surgeries performed at Barts Health NHS trust means that a homogenous population of specific surgeries (for instance high-risk, major abdominal surgery) can be recruited in a short period of time.

Due to funding from the Barts Health Comprehensive Local Research Network (CLRN), as well as from other independent funding streams, there is a cohort of highly skilled research nurses who screen, consent, take bloods and follow up the patients for the duration of their admission. As all surgery is elective, there are not the same consent issues and a timely informed consent can be obtained prior to surgery.

6.24 Experimental Methodologies

Due to the symbiotic relationship between Barts Health NHS trust and Bart & the London School of Medicine & Dentistry the transfer of clinical samples, technical analysis, as well as scientific advice was available by collaboration with core centres or other research groups. For the genomic analysis prior to extraction of total RNA conversations took place with the Genome Centre, William Harvey Research Centre as well as Professor Julian C Knight's Group at the Wellcome Trust Centre for Human Genomics, Oxford as to best research practice. This allowed us to extract total RNA from all samples, permitting mRNA analysis as well as later (ongoing) analysis of smaller non-coding RNA for epigenetic analysis. Gene expression with the Taqman system is a well-validated and reproducible protocol, as the 384 well plate preparation was carried out by robot, which also minimised pipetting error. The analysis of protein concentration via ELISA assay was performed in a well-validated, reproducible manner using off the shelf consumables. The *ex vivo* flow cytometry was performed using an

international standardised protocol that is widely used in the published literature as a marker of immune competence. Due to the study design in the perioperative cohort, with each patient acting as their own control, this allowed accurate inferences to be made when incubating pre and postoperative serum on pooled healthy donor PBMCs. This then accounted for their individual premorbid comorbidities such as the preoperative diagnosis of cancer, diabetes or preoperative immunosuppression via paired statistical analysis. In the trauma cohort the use of a healthy control population with whole blood total RNA gathered in the same manner provided a valid comparator. Although this required the use of a separate set of ethics, this, we felt, provided a more robust control than recruiting minor injured patients (ISS<4) who will still have a degree of systemic immune activation following injury.

6.25 Statistical Analysis

Statistical analysis was carried out by myself and Dr Michael J O'Dwyer (primary Ph.D. supervisor) with consultation from the perioperative medicine group in-house statistician Ms Tahania Ahmad and Dr John R Prowle, a renal intensivist with a comprehensive statistical background. Missing data is an unfortunate hazard of clinical research, however every effort was made by me to minimise this by requesting and reviewing the original patient case notes, allowing a more complete data set to be analysed. In the case of the trauma cohort, due to the severity of the injuries that these patients suffered a small minority, 9 patients (8%), did not survive to the 24 or 72 hour sampling time points. As one of the primary aims of the thesis was to examine the acute changes in gene expression on targeted T_h cell inflammatory pathways following polytrauma it was important that all eligible patients were included. In the case of the perioperative cohort the data set and sample bio-bank was much more complete.

Variables were analysed preferentially as continuous variables without dichotomising them. If variables were dichotomised then these were based on standardised definitions such as the presence of shock on admission (Davis *et al.*, 1991; Spahn *et al.*, 2013) or the presence of MODS (Moreno *et al.*, 1999). For the construction of multivariate models, univariate analysis

was performed on all variables thought likely to play a clinical role in the process, with those recording a *P*-value of <0.1 being included in the model. All *P*-values were reported as un-corrected to allow comparison between the differing models in each Chapter.

6.3 Weaknesses Of This Thesis

6.31 Introduction

The limitations of each individual Chapter have been discussed in the relevant sections, however there are some broad overarching weaknesses that require further clarification.

6.32 Study Design In The Trauma Cohort

ACIT2 is a study that was primarily designed to examine the hyper-acute and acute effects of ATC and Trauma Induced Coagulopathy (TAC). As a consequence the sampling times, as well as the samples that are collected, are designed primarily address these coagulopathy-based research questions. Following the results generated from this thesis and the more in depth genomic analysis that is ongoing there is a move to alter the blood draw to initially; capture the immunological response more comprehensively with the isolation PBMCs for flow cytometry analysis in order to undertake *in vitro* work on the collection of serum (rather than citrated plasma) for cell based models. There is also a move towards the implementation of later blood sampling points in order to capture more accurately the changes to the adaptive arm of immune system and more closely monitor the immune systems of patients when they are suffering from persistent MODS on the critical care unit.

As has been demonstrated in the perioperative aspects of this thesis, tissue damage in the absence of shock profoundly affects the human immune system. In the introduction of this thesis our current understanding of the consequences of general anaesthesia, temperature control as well as the influences of the numerous therapeutics that are administered during this period as well as on the critical care unit and their affect on the immune response are outlined. As a consequence when following up these patients more of the clinical research form should

be dedicated to recording these influences allowing later research to address more holistically the inflammatory paradigm.

6.33 Study Design In The Perioperative Cohort

In the perioperative cohort, due to the fact that samples were drawn from patients enrolled in a study designed to detect postoperative rises in Troponin T (Vascular Events In Noncardiac Surgery Patients Cohort Evaluation Study *et al.*, 2012), these pre-defined sampling time points meant that we were unable to examine the hyper-acute response to major abdominal surgery. As a consequence, on analysing these limited time points we may have missed hyper-acute rises in the assayed genes, such as the IFN-γ peak that was seen in the trauma cohort. However, following on from these data, a study designed specifically to examine the relationship between immunological changes following major abdominal surgery and the acquisition of late nosocomial infection began recruiting in May 2015. To date this has recruited over 100 patients, with the first 50 patients being sampled at a hyper-acute 2-6 hour time period following abdominal closure. We await the analysis of these data. The instigation of a study purely designed to address inflammation means that similar concerns that were discussed with the ACIT2 study, such as sample types, are addressed in this cohort

6.33 Data Collection

In the context of an observational study a balance has to be met with a level of data that can be pragmatically (accurately) collected and the utility of the data. As has already been highlighted in this thesis the research discussed here was carried out as two sub-studies of two large international observational studies not primarily designed to address the immunological consequences of tissue damage or shock. As a result as discussed in the introduction of this thesis, the immune system is clearly influenced by a multitude of factors with a number of these factors perhaps not adequately represented with the current data collection constraints. Some clear aspects that require better clarification and documentation in the future are the choice of anaesthesia (in both elective major abdominal surgery and semi-elective (or

emergency) trauma surgery) and transfusion triggers for perioperative transfusion as well as transfusion on the critical care unit as these are aspects of care that are not protocolled and as a result are left to the discretion of the consultant anaesthetist or intensivist. This is in stark contrast to the pre-hospital care that trauma patients receive which is heavily standardised and protocolled. Other factors that have been added to the clinical research form for the novel study include measures of temperature, a preoperative risk score and the use of perioperative dexamethasone or catecholamines. In both cohorts the value of neutrophil: lymphocyte ratios and neutrophil: monocyte ratio was assessed as this had been suggested as a valuable predictor of poor outcome in both perioperative cohorts and trauma cohorts. Unfortunately no signal was detected, this may be in part due to the low patient numbers when compared to previously published studies (Salciccioli *et al.*, 2015).

Outcome scoring is another aspect that can be improved upon. It is now simply not enough to report mortality or length of stay in RCTs so more detail is clearly required in hypothesis generating observational studies. The recording of organ dysfunction particularly in trauma is important but its measurement is complicated by an often-coexisting TBI. In order to maximize cerebral perfusion pressure thus aiming to prevent secondary brain injury patients often will be placed on large doses of noradrenaline to drive up their MAP. This means that when using SOFA as a measure of organ dysfunction this will lead to a skewing of the scores as the patient may have no cardiovascular organ failure (as assessed by MAP and noradrenaline dose, outlined in Supplementary Table 3) yet will be scored incorrectly as having not only a single organ failure (TBI) but also suffering from multi organ failure. The scoring of lung injury is again an aspect that can be improved upon in the context of trauma patients. As has been previously discussed, with the preponderance of western trauma being blunt, many patients will present with pneumothoraces, haemothoraces or lung contusions or a combination of all of three of these pathologies. This then makes the interpretation of chest radiographs and CTs problematic, as it is not clear if infiltrates are secondary to the initial traumatic injury or secondary to a later lung injury. In the thesis due to the limited data collected on lung injury

routinely by the trauma group a decision was made to characterise patients as having the presence or absence of ARDS through their respiratory SOFA score, essentially their P_aO_2 F_iO_2 (P/F) ratio with those recording a P/F ratio of ≤26.7 (kPa) for a period of ≥48 hours being classified as having ARDS. The limitations of this as an accurate measure of true ARDS in the absence of the classification via the Berlin definition is acknowledged. In future studies data collection has improved in order to fully classify patients as mild, moderate or severe ARDS based on the much more robust Berlin criteria (Force *et al.*, 2012).

6.34 Experimental Techniques

Due to funding constraints at the start of the project concessions were made regarding the extent of the initial genomic analysis, which was to be performed on the samples available. As a consequence a more frugal targeted approach was selected, examining a series of markers, which were felt to represent temporal T-helper cell differentiation. If greater amounts of funding had been available initially a more systematic approach would have been favoured either utilising a targeted gene array chip to examine a more complete selection of adaptive genes, a genome wide array in the same manner as utilised in the genomic storm paper (Xiao *et al.*, 2011) or RNA sequencing techniques. Interestingly, following the successful genomic data outlined in this thesis, funding was made available to carry out genome wide gene expression using an array chip on a number of the mRNA samples isolated from patients included in this body of work. Further details regarding this are briefly outlined in the further work aspect of this Chapter. With the commencement of a novel study aiming to describe the temporal immunological response following major abdominal surgery a more systematic quantification of gene expression will also be possible in the perioperative cohort allowing novel analysis of the hyper-acute inflammatory changes with the 2-6 hour postoperative sampling point.

PAXgene® tubes are a highly reliable product for the collection and storage of whole blood total RNA, however they are limited by what they are actually collecting, whole blood total RNA. By the normalisation of candidate gene PCR expression data to reference genes

which are demonstrated to be expressed at stable levels in these patients we were able to correct for the effect of relative changes in the total leukocyte population in individual whole blood mRNA samples (Vandesompele et al., 2002). However, due to the fact that there are disparities in the differential leukocyte count, such as 24 and 72 hour sampling points, a neutrophilia and simultaneous lymphopenia, we cannot exclude the possibility that the observed changes in mRNA levels are explained, at least in part, by alterations in the relative abundance of specific leucocyte subpopulations collected in the whole blood samples from which the RNA was extracted for this study (Laudanski *et al.*, 2006). A paired full blood counts sample is sent to the clinical laboratory with each blood draw, which gives data on the differential leukocyte count. In an attempt to account for the potential lymphopenia it was discussed as to whether there was validity in dividing the gene expression values by the paired lymphocyte count, of those lymphocyte specific cytokines and transcription factors, in an attempt to obtain a gene expression value independent of any co-existing lymphopenia. Despite extensive discussions with colleagues and literature searches we have been unable to find a study that utilises this methodology and validates this potential methodology. In the case of the ongoing analysis of whole blood mRNA genome wide gene expression array in order to account for the variability of the leukocyte subpopulations a novel algorithm is employed (Abbas *et al.*, 2005; Zhong *et al.*, 2013). This algorithm aims to extract cell-specific gene expression signatures allowing whole blood mRNA or mixed tissue samples to be analysed as though they had been FACS sorted. This is an exciting and novel experimental technique.

6.4 Further Work

In order to continue this research stream data generated from this thesis and from ongoing postdoctoral work has been used in the application for grants to further both the trauma and perioperative research.

6.4.1 Trauma Research

6.4.1.1 Genomics

As has been previously alluded to there have been expansions in the genomic research streams due to the promising pilot data generated as part of this thesis. A more homogenous cohort of patients with or without multi-organ dysfunction, all suffering blunt polytrauma in the absence of TBI, have undergone genome wide gene expression array (Illumina HumanHT-12 v4 Expression BeadChip) analysis in order to examine more completely the temporal peripheral immunological response. These data describe a focused leukocyte response involving only 4% of the transcriptome within the hyper-acute time window, rapidly blossoming to a full 20% of the whole leukocyte genome at 24 hours, when compared to controls with minor injuries (ISS<4). On further analysis, utilising the IRIS system (Abbas *et al.*, 2005), demonstrated changes in innate lymphoid cell populations, specifically NK cells. As a result ongoing collaborative work led by Dr Joanne Shepherd will focus on the mechanistic role that NK cells play in the pathology of protracted multi-organ dysfunction.

6.4.1.2 Epigenomics

As total RNA was extracted from the PAXgene® tube, this permitted not only the analysis of mRNA but also non-coding RNAs such as miRNAs. In order systematically to characterise the expression levels of both circulating and intracellular miRNAs following severe polytrauma miRNA 100 patients were selected at two time points (0 and 24 hours), 50 suffering a pneumonia and 50 not. miRNA sequencing was performed using the Illumina HiSeq2500. Following this the sequences were aligned to the human GRCh37 reference genome with functional enrichment analysis performed using Ingenuity Pathway Analysis on all miRs reaching an adjusted p value of <0.1. The complementary mRNA targets of interest were identified using miRBase and TargetScan (www.mirbase.org, www.targetscan.org). Ingenuity Pathway Analysis highlighted Cancer, Haematological Disease, Immunological and Inflammatory Disease as well as Organismal Injury and Abnormalities as important pathways

altered between infected and non-infected patients. A more in-depth bioinformatics analysis as ongoing directed by Dr Michael Barnes and Dr Timothy F Jones.

In addition to this there is ongoing work examining the role of genome wide methylation of peripheral leukocytes and the influence that this has in enhancing the susceptibility to nosocomial infection.

6.4.1.3 Flow Cytometry

Following the description of the acute changes in mHLA-DR expression resulting from major abdominal surgery a cohort of 12 consecutive patients requiring ICU admission were recruited to identify if similar changes occurred following severe polytrauma.

In addition to the characterisation of mHLA-DR expression on a larger cohort of trauma patients a systematic approach to the immunophenotyping and sorting of peripheral leukocytes has commenced. The panels are based on proposed methodologies to standarise the markers used for the identification of human leukocytes (Maecker *et al.*, 2012). This Work is being continued by Dr Joanne Shepherd.

6.4.1.4 *In Vitro* Work

Work is on going to replicate the findings outlined in Chapter five, but in a severe polytrauma cohort. Healthy $CD14^{+}$ cultured cells are additionally being sorted following flow cytometry (mHLA-DR) interrogation to allow later downstream targeted gene expression analysis after culture in the differing environments described in Chapter five. There is also the potential to assess the influence of trauma or perioperative PBMCs, their response to either healthy control serum to see if they spontaneously re-express HLA-DR molecules after removal from the proposed immunosuppressive environment as well as culture with immune stimulants to assess reversibility of patient monocytes or PBMCs *in vitro*.

6.4.2 Perioperative Research

6.4.2.1 Biomarker Based Identification of Nosocomial Infectious Complications (BIONIC)

As had been alluded to earlier in this Chapter a novel observational study has commenced recruiting elective major abdominal surgical patients at the RLH since May 2015. The inclusion criteria is similar to the cohort of patients analysed in Chapters four and five of this thesis, with the first 50 patients recruited undergoing sampling at 2-6 hour point, following abdominal closure. This will allow capture of the hyper-acute inflammatory response that was lacking in the previous investigations described in this thesis. In addition to the routinely collected citrated plasma, buffy coats, serum and PAXgene® tubes, EDTA anticoagulated blood was drawn and stained <one hour from blood draw for the immediate quantification of mHLA-DR expression pre and postoperatively. The interim data analysis of the first 80 patients recruited demonstrated that initially there were marked changes in the mHLA-DR expression levels as early as 2-6 hours postoperatively. At 24 hours there was a difference between those who went on to acquire a postoperative nosocomial infection and those who remained infection free. This phenomenon became more profound at the 48 hour sampling time point.

6.5 Conclusions

This thesis has completed all the previously defined aims. It has provided novel experimental data; with large amounts already being published in leading speciality peer reviewed journals. It describes a significant host immune response, which occurs immediately following significant tissue damage that is dominated by features of immune suppression. Allogeneic blood transfusion appears to have a distinct, additive effect in both severe polytrauma and perioperative care. Finally the thesis finally identifies a potential role for targeted treatment with currently licenced immune stimulants (IFN-γ and GM-CSF) as well as the potential for the exploitation of the IL-10 signalling pathway in reducing the incidence of

nosocomial infections. Work is continuing in both the fields of trauma and perioperative medicine to further build on this thesis.

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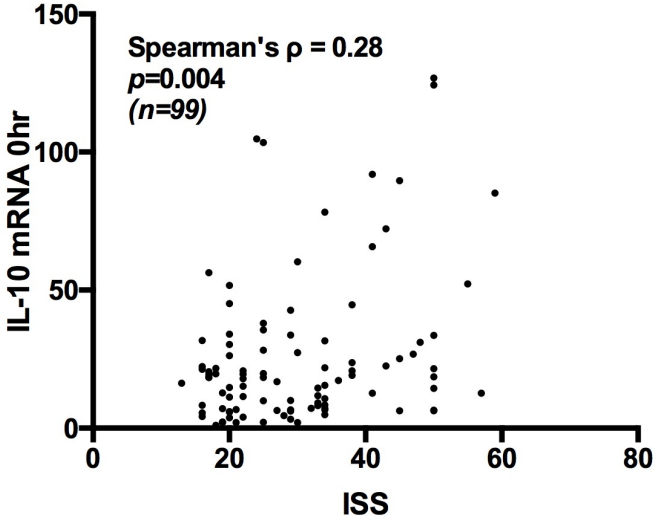
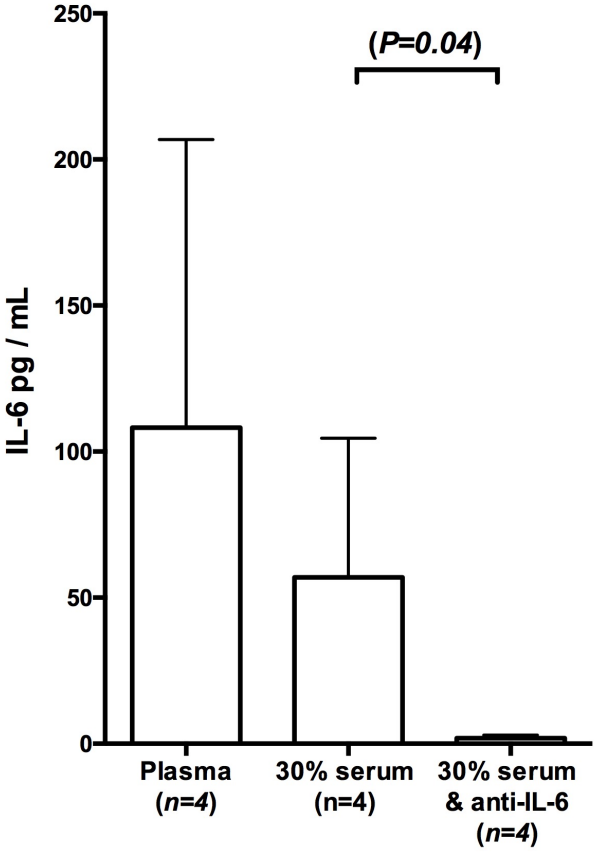
Appendixes

Supplementary Figures



Supplementary Figure 1 - The relationship between IL-10 expression and injury severity (ISS)

All results are expressed as a relative quantification ratio between candidate and reference genes.



Supplementary Figure 2 - Efficacy of IL-6 blocking antibody

Changes in IL-6 protein concentration when postoperative serum was cultured in the presence or absence of an IL-6 blocking antibody. All data displayed as median and interquartile range.

Supplementary Tables

Supplementary Table 1 - Screening protocols for ACIT2

Exclusion criteria
E1 The patient is <16 years old
E2 The patient has been transferred from another hospital
E3 More than 2 hours has elapsed from the time of injury
E4 Received more than 2000ml fluids
E5 Patient has suffered >5% burns BSA
E6 Known severe liver disease
E7 Known bleeding abnormality/coagulation drugs
E8 The trauma team leader deems the patient inappropriate for the trial
Other reason(s) why the patient was not recruited
R1 Unable to attend call
R2 Minor injuries, normal 1^0 survey, d/c within 24-hrs
R3 Non-trauma
R4 Unable to gain access for blood sample
R5 Other reason (specify)

Supplementary Table 2 - Calculation of the Injury Severity Score (ISS)

Anatomical Regions	Abbreviated Injury Score (AIS) Grading of Severity	Injury Severity Score
Head & Neck Face Chest Abdominal or pelvis contents Extremities or pelvic girdle External	1 – Minor 2 – Moderate 3 – Severe (Non life-threatening) 4 – Severe (Life-threatening) 5 – Critical (Survival uncertain)	Taking the top three AIS scores, square each one and add them together to obtain a score out of 75

Supplementary Table 3 - Calculation of the Sequential Organ Failure Assessment (SOFA) score

SOFA Score	0	1	2	3	4
PaO2 / FiO2 (kPa)	>53.3	≤53.3	≤40	≤26.7	≤13.3
Platelets	≥150	<150	<100	<50	<20
Bilirubin µmol/L	<20	20-32	33-101	102-204	>204
CVS	MAP≥70	MAP<70	Dopamine ≤5 or any dose of dobutamine	Dopamine >5 or adrenaline ≤0.1 or noradrenaline ≤0.1 mcg/kg/min	Dopamine >15 or adrenaline >0.1 or noradrenaline >0.1 mcg/kg/min
GCS	15	13-14	10-12	6-9	<6
Creatinine µmol/L	<109	110-170	171-299	300-440 or <500ml/day of urine	>440 or <200ml/day of urine

Supplementary Table 4– Criteria for defining sites of infection

Infection site	Definition
BSI – Bloodsteam Infection (LCBI - Laboratory-Confirmed Bloodstream Infection, Secondary BSI- Secondary Bloodstream Infection)	**Laboratory-Confirmed Bloodstream Infection (LCBI)** must meet at least 1 of the following criteria: 1. Patient has a recognized pathogen cultured from 1 or more blood cultures. **AND** organism cultured from blood is not related to an infection at another site. 2. Patient has at least 1 of the following signs or symptoms: fever (>38°C), chills, or hypotension. **AND** signs and symptoms and positive laboratory results are not related to an infection at another site. **AND** common skin contaminant is cultured from 2 or more blood cultures drawn on separate occasions. **Secondary Bloodstream Infection (BSI)** In a patient suspected of having an infection, blood and a site-specific specimen are collected for culture and both are positive for at least one matching organism. If the site-specific culture is an element used to meet the infection site criterion, then the BSI is considered secondary to that site-specific infection.
Pneumonia	**Pneumonia** requires any 1 of the following: 1. Rales or dullness to percussion on physical examinations of chest **AND** any of the following: A. New onset of purulent sputum or change in character of sputum. B. Isolation of organism from blood culture. C. Isolation of pathogen from specimen obtained by trans-tracheal aspirate, bronchial brushing, or biopsy. 2. Chest radiography showing new or progressive infiltrate, consolidation, cavitation, or pleural effusion. **AND** any of the following: A. New onset of purulent sputum or change in character of sputum. B. Isolation of organism from blood culture. C. Isolation of pathogen from specimen obtained by trans-tracheal aspirate, bronchial brushing, or biopsy. D. Isolation of virus or detection of viral antigen in respiratory secretions. E. Diagnostic single antibody titre (IgM) or fourfold increase in paired serum samples (IgG) for pathogen. F. Histopathologic evidence of pneumonia.

Surgical Site infection (SIS-Superficial incisional surgical site infection, DIS-Deep incisional surgical site infection)	**A superficial incisional SSI** must meet the following criterion: Infection occurs within 30 days after the operative procedure. **AND** involves only skin and subcutaneous tissue of the incision. **AND** patient has at least *1* of the following: A. Purulent drainage from the superficial incision. B. Organisms isolated from an aseptically obtained culture of fluid or tissue from the superficial incision. C. At least *1* of the following signs or symptoms of infection: pain or tenderness, localized swelling, redness, or heat, *and* superficial incision is deliberately opened by surgeon and is culture positive or not cultured. A culture-negative finding does not meet this criterion. D. Diagnosis of superficial incisional SSI by the surgeon or attending physician. **A deep incisional SSI** must meet the following criterion: Infection occurs within 30 days after the operative procedure if no implant is left in place. **AND** involves deep soft tissues (eg, fascial and muscle layers) of the incision. **AND** patient has at least *1* of the following: A. Purulent drainage from the deep incision but not from the organ/space component of the surgical site. B. A deep incision spontaneously dehisces or is deliberately opened by a surgeon and is culture-positive or not cultured when the patient has at least *1* of the following signs or symptoms: fever (>38°C), or localized pain or tenderness. A culture-negative finding does not meet this criterion. C. An abscess or other evidence of infection involving the deep incision is found on direct examination, during reoperation, or by histopathologic or radiologic examination. D. Diagnosis of a deep incisional SSI by a surgeon or attending physician.
Urinary tract infection (SUTI)	**A symptomatic urinary tract infection** must meet at least *1* of the following criteria: 1. Patient has at least *1* of the following signs or symptoms with no other recognized cause: fever (>38°C), urgency, frequency, dysuria, or suprapubic tenderness. **AND** patient has a positive urine culture, that is, $\geq 10^5$ microorganisms per cc of urine with no more than 2 species of microorganisms. 2. Patient has at least *2* of the following signs or symptoms with no other recognized cause: fever (>38°C), urgency, frequency, dysuria, or suprapubic tenderness. **AND** at least *1* of the following: A. Positive dipstick for leukocyte esterase and/or nitrate. B. Pyuria (urine specimen with ≥10 white blood cell [WBC]/mm^3 or ≥3 WBC/high-power field of unspun urine). C. Organisms seen on Gram's stain of unspun urine. D. At least *2* urine cultures with repeated isolation of the same uropathogen (gram-negative bacteria or *Staphylococcus saprophyticus*) with $\geq 10^2$ colonies/mL in nonvoided specimens. E. $\leq 10^5$ colonies/mL of a single uropathogen (gram-negative bacteria or *S saprophyticus*) in a patient being treated with an effective antimicrobial agent for a urinary tract infection. F. Physician diagnosis of a urinary tract infection. G. Physician institutes appropriate therapy for a urinary tract infection.

Intra-abdominal infection (Organ/space-Organ/space surgical site infection, IAB-Intra-abdominal, not specified elsewhere)	**Organ/space-Organ/space surgical site infection** must meet the following criterion: Infection occurs within 30 days after the operative procedure if no implant is left in place or within 1 year if implant is in place and the infection appears to be related to the operative procedure. **AND** infection involves any part of the body, excluding the skin incision, fascia, or muscle layers, that is opened or manipulated during the operative procedure. **AND** patient has at least *1* of the following: A. Purulent drainage from a drain that is placed through a stab wound into the organ/space. B. Organisms isolated from an aseptically obtained culture of fluid or tissue in the organ/space. C. An abscess or other evidence of infection involving the organ/space that is found on direct examination, during reoperation, or by histopathologic or radiologic examination. D. Diagnosis of an organ/space SSI by a surgeon or attending physician. **IAB-Intra-abdominal, not specified elsewhere** including gallbladder, bile ducts, liver (excluding viral hepatitis), spleen, pancreas, peritoneum, subphrenic or subdiaphragmatic space, or other intraabdominal tissue or area not specified elsewhere must meet at least *1* of the following criteria: 1. Patient has organisms cultured from purulent material from intraabdominal space obtained during a surgical operation or needle aspiration. 2. Patient has abscess or other evidence of intra-abdominal infection seen during a surgical operation or histopathologic examination. 3. Patient has at least *2* of the following signs or symptoms with no other recognized cause: fever (>38°C), nausea, vomiting, abdominal pain, or jaundice. **AND** at least *1* of the following: A. Organisms cultured from drainage from surgically placed drain (eg, closed suction drainage system, open drain, T-tube drain). B. Organisms seen on Gram's stain of drainage or tissue obtained during surgical operation or needle aspiration. C. Organisms cultured from blood *&* radiographic evidence of infection (eg, abnormal findings on ultrasound, CT scan, MRI, or radiolabel scans [gallium, technetium, etc] or on abdominal x-ray).
Intra vascular catheter related infection (VASC-Arterial or venous infection)	**Arterial or venous infection** must meet at least 1 of the following criteria: 1. Patient has organisms cultured from arteries or veins removed during a surgical operation. **AND** blood culture not done or no organisms cultured from blood. 2. Patient has evidence of arterial or venous infection seen during a surgical operation or histopathologic examination. 3. Patient has at least 1 of the following signs or symptoms with no other recognized cause: fever (>38°C), pain, erythema, or heat at involved vascular site. **AND** more than 15 colonies cultured from intravascular cannula tip using semiquantitative culture method. **AND** blood culture not done or no organisms cultured from blood. 4. Patient has purulent drainage at involved vascular site. **AND** blood culture not done or no organisms cultured from blood.

Skin infection (SKIN)	**Skin infections** must meet at least *1* of the following criteria: 1. Patient has purulent drainage, pustules, vesicles, or boils. 2. Patient has at least *2* of the following signs or symptoms with no other recognized cause: pain or tenderness, localized swelling, redness, or heat. **AND** at least *1* of the following: A. Organisms cultured from aspirate or drainage from affected site; if organisms are normal skin flora they must be a pure culture. B. Organisms cultured from blood. C. Positive antigen test performed on infected tissue or blood (eg, herpes simplex, varicella zoster, *H influenzae*, *N meningitidis*). D. Multinucleated giant cells seen on microscopic examination of affected tissue. E. Diagnostic single antibody titer (IgM) or 4-fold increase in paired sera (IgG) for pathogen.

Supplementary Table 5 - Univariate analysis for associations with gene expression following polytrauma

Response Variable	Immediate Transfusion	Early Transfusion	Number of Units	*s*SBP	Lactate	BE	ISS	TBI	Age
IL-10 ***0HR***	0.003	N/A	N/A	0.11	0.5	0.004	0.02	0.2	0.47
IL-10 ***24HR***	0.014	0.0001	0.0001	.0.4	0.0001	0.0001	0.14	0.03	0.97
RORγt ***24HR***	0.17	0.05	0.016	0.25	0.08	0.1	0.66	0.1	0.22
Δ TNF-α ***0-24HR***	0.0197	0.015	0.05	0.043	0.61	0.6	0.61	0.02	0.006
Δ INFγ ***0-24HR***	0.0062	0.035	0.0002	0.14	0.0001	0.0001	0.13	0.008	0.9
Δ T-bet ***0-24HR***	0.15	0.03	0.0008	0.037	0.0001	0.0001	0.23	0.057	0.67

Δ, Signifies dynamic variables, as change in cytokine expression over time period shown. *BE*, time 0HR base excess. *Early Transfusion,* transfusion within the first 24HR of injury. *Immediate Transfusion,* transfusion of PRBC prior to 0HR blood draw. *ISS,* Injury Severity Score. *Lactate,* time 0HR Lactate. *N/A*, not applicable. *Number of Unit*s, number of units of PRBC transfused in first 24HR post-injury. *sSBP,* first at scene systolic blood pressure. *TBI*, traumatic brain injury.

Supplementary Table 6 - Multivariate regression analysis for prediction of gene expression following polytrauma

Response Variable	Predictor Variables Included in the model									Whole Model R^2	Predictors Independently Associated With Response Variable								
	Immediate Transfusion	Early Transfusion	Number of Units	*s*SBP	Lactate	BE	ISS	TBI	Age		Immediate Transfusion	Early Transfusion	Number of Units	*s*SBP	Lactate	BE	ISS	TBI	Age
IL-10 *0HR*	***x***					***x***	***x***			**0.16**	**0.04**						**0.005**		
IL-10 *24HR*	***x***	***x***	***x***		***x***	***x***		x		**0.44**	**.09**		**<0.0001***			**0.04**			
RORγt *24HR*		***x***	***x***		***x***	***x***				**0.09**			**0.01***						
Δ TNF-α *0-24HR*	***x***	***x***	***x***	***x***				x	x	**0.39**	**0.08**	**0.05**		**0.01**					**0.0008**
Δ INFγ *0-24HR*	***x***	***x***	***x***		***x***	**x**		x		**0.004**		**0.006***							
Δ T-bet *0-24HR*		***x***	***x***	***x***	***x***	***x***		x		**0.07**		**0.7***			**0.06**				

Multivariate regression analysis of gene expression using transfusion requirements, early blood pressure measurements and selected markers of tissue ischaemia and tissue perfusion. Each **x** represents an individual variable included in the model. These univariate analyses are presented in a Table 4. Selection of variables is described in the methods section. The numbers under '*Predictors Independently Associated With Response Variable'* represent the *p*-values obtained when the model was run and are indicative of an independent association with the response variable. An empty cell implies that no independent association was detected. **,* Indicates *p*-value obtained following backwards elimination. **Δ**, Signifies dynamic variables, as change in cytokine expression over time period displayed. *BE*, time 0HR base excess. *Early Transfusion,* transfusion within the first 24HR of injury. *Immediate Transfusion,* transfusion of PRBC prior to 0HR blood draw. *ISS,* Injury Severity Score. *Lactate,* time 0HR Lactate. *Number of Unit*s, number of units of PRBC transfused in first 24HR post-injury. *sSBP,* first at scene systolic blood pressure. *TBI*, traumatic brain injury.

Supplementary Table 7 – Incidence of Blood Stream Infection following polytrauma

Organism	Number
Acinetobacter baumannii	4
Bacillus cereus	1
Bacteroides fragilis	1
Candida albicans	2
Enterobacter cloacae	2
Escherichia coli	3
Klebsiella pneumonia	1
Moraxella catarrhalis	1
Pseudomonas aeruginosa	2
Staphylococcus aureus	3

All positive blood cultures were reviewed in conjunction with the microbiology department and likely contaminants were excluded from the subsequent analysis. BSI, blood stream infection encompassing bacteraemias and fungaemias. Patients recorded multiple BSIs as a result all infective organisms cultured have been recorded.

Supplementary Table 8 - Multivariate regression analysis for prediction of gene expression following polytrauma

Response Variable	Predictor Variables on Univariate Analysis for the Model						Whole Model	Predictors Independently Associated With Response Variable					
	Median age of blood	Quantity of PRBCs transfused	Baseline BD	24HR BD	ISS	Age		Median age of blood	Quantity of PRBCs transfused	Baseline BD	24HR BD	ISS	Age
Δ RORγt 24 / 0 HR	$r^2 = 0.19$ $P = 0.003$	$r^2 = 0.09$ $P = 0.05$	$r^2 = 0.07$ $P = 0.09$	$r^2 = 0.009$ $P = 0.58$	$r^2 = 0.02$ $P = 0.37$	$r^2 = 0.07$ $P = 0.06$	$r^2 = 0.28$ $P = 0.01$	$P = 0.01$	$P = 0.36$	$P = 0.46$	*x*	*x*	$P = 0.40$
Δ IL-12 24 / 0 HR	$r^2 = 0.10$ $P = 0.03$	$r^2 = 0.03$ $P = 0.26$	$r^2 = 0.05$ $P = 0.16$	$r^2 = 0.03$ $P = 0.33$	$r^2 = 0.009$ $P = 0.52$	$r^2 = 0.09$ $P = 0.04$	$r^2 = 0.15$ $P = 0.04$	$P = 0.05$*	*x*	$P = 0.23$*	*x*	*x*	$P = 0.25^{\dagger}$
Δ IL-23 24 / 0 HR	$r^2 = 0.10$ $P = 0.03$	$r^2 = 0.04$ $P = 0.19$	$r^2 = 0.03$ $P = 0.30$	$r^2 = 0.003$ $P = 0.74$	$r^2 = 0.007$ $P = 0.74$	$r^2 = 0.17$ $P = 0.005$	$r^2 = 0.22$ $P = 0.01$	$P = 0.29$	$P = 0.16$	x	x	x	$P = 0.11$
Δ FOXP3 24 / 0 HR	$r^2 = 0.09$ $P = 0.04$	$r^2 = 0.0002$ $P = 0.93$	$r^2 = 0.0003$ $P = 0.91$	$r^2 = 0.03$ $P = 0.36$	$r^2 = 0.002$ $P = 0.78$	$r^2 = 0.14$ $P = 0.01$	$r^2 = 0.16$ $P = 0.02$	$P = 0.24$	*x*	*x*	*x*	*x*	$P = 0.06$
Δ GATA-3 24 / 0 HR	$r^2 = 0.16$ $P = 0.007$	$r^2 = 0.02$ $P = 0.39$	$r^2 = 0.01$ $P = 0.47$	$r^2 = 0.008$ $P = 0.60$	$r^2 = 0.00003$ $P = 0.97$	$r^2 = 0.21$ $P = 0.002$	$r^2 = 0.26$ $P = 0.002$	$P = 0.08$	*x*	*x*	*x*	*x*	$P = 0.02$
Δ IL-12 72 / 0 HR	$r^2 = 0.21$ $P = 0.01$	$r^2 = 0.04$ $P = 0.27$	$r^2 = 0.004$ $P = 0.75$	$r^2 = 0.007$ $P = 0.70$	$r^2 = 0.02$ $P = 0.48$	$r^2 = 0.12$ $P = 0.07$	$r^2 = 0.24$ $P = 0.03$	$P = 0.05$	*x*	*x*	*x*	*x*	$P = 0.31$
Δ TGF-β 72 / 0 HR	$r^2 = 0.16$ $P = 0.03$	$r^2 = 0.006$ $P = 0.69$	$r^2 = 0.008$ $P = 0.65$	$r^2 = 0.01$ $P = 0.58$	$r^2 = 0.006$ $P = 0.90$	$r^2 = 0.04$ $P = 0.27$	$r^2 = 0.16$ $P = 0.03$	$P = 0.03$	*x*	*x*	*x*	*x*	*x*

Multivariate regression analysis of gene expression using median age of blood transfused, transfusion requirement, selected markers of tissue injury, tissue ischaemia and age. Each univariate *P*-value and r^2 value is displayed. *, Indicates *P*-value obtained following backwards elimination. †, Indicates *P*-value obtained prior to exclusion via backward elimination. **Δ**, Indicates dynamic variables, as change in cytokine expression over time period displayed. *BD*, base deficit (at baseline (admission) and 24HR (24HR after injury). *ISS,* Injury Severity Score. *Quantity of PRBCs transfused*, number of units of packed red blood cells (PRBCs) transfused in first 24HR post-injury. *X,* Indicates that univariate analysis did not meet threshold of $P < 0.25$ for inclusion in multivariate regression analysis model.

Supplementary Table 9 - Multivariate Regression Analysis: With all variables added to each response variable for each model

Response Variable	Predictor Variables on Univariate Analysis for the Model						Whole Model	Variable					
	Median age of blood	Quantity of PRBCs transfused	Baseline BD	24HR BD	ISS	Age		Median age of blood	Quantity of PRBCs transfused	Baseline BD	24HR BD	ISS	Age
Δ RORγt 24 / 0 HR	$r^2 = 0.19$	$r^2 = 0.09$	$r^2 = 0.07$	$r^2 = 0.009$	$r^2 = 0.02$	$r^2 = 0.07$	$r^2 = 0.31$	$P = 0.06$	$P = 0.23$	$P = 0.79$	$P = 0.97$	$P = 0.48$	$P = 0.63$
	$P = 0.003$	$P = 0.05$	$P = 0.09$	$P = 0.58$	$P = 0.37$	$P = 0.06$	$P = 0.07$						
Δ IL-12 24 / 0 HR	$r^2 = 0.10$	$r^2 = 0.03$	$r^2 = 0.05$	$r^2 = 0.03$	$r^2 = 0.009$	$r^2 = 0.09$	$r^2 = 0.20$	$P = 0.37$	$P = 0.76$	$P = 0.13$	$P = 0.45$	$P = 0.30$	$P = 0.13$
	$P = 0.03$	$P = 0.26$	$P = 0.16$	$P = 0.33$	$P = 0.52$	$P = 0.04$	$P = 0.33$						
Δ IL-23 24 / 0 HR	$r^2 = 0.10$	$r^2 = 0.04$	$r^2 = 0.03$	$r^2 = 0.003$	$r^2 = 0.007$	$r^2 = 0.17$	$r^2 = 0.26$	$P = 0.42$	$P = 0.31$	$P = 0.93$	$P = 0.91$	$P = 0.47$	$P = 0.12$
	$P = 0.03$	$P = 0.19$	$P = 0.30$	$P = 0.74$	$P = 0.74$	$P = 0.005$	$P = 0.16$						
Δ FOXP3 24 / 0 HR	$r^2 = 0.09$	$r^2 = 0.0002$	$r^2 = 0.0003$	$r^2 = 0.03$	$r^2 = 0.002$	$r^2 = 0.14$	$r^2 = 0.20$	$P = 0.40$	$P = 0.38$	$P = 0.67$	$P = 0.66$	$P = 0.86$	$P = 0.20$
	$P = 0.04$	$P = 0.93$	$P = 0.91$	$P = 0.36$	$P = 0.78$	$P = 0.01$	$P = 0.02$						
Δ GATA-3 24 / 0 HR	$r^2 = 0.16$	$r^2 = 0.02$	$r^2 = 0.01$	$r^2 = 0.008$	$r^2 = 0.00003$	$r^2 = 0.21$	$r^2 = 0.32$	$P = 0.15$	$P = 0.27$	$P = 0.85$	$P = 0.90$	$P = 0.81$	$P = 0.12$
	$P = 0.007$	$P = 0.39$	$P = 0.47$	$P = 0.60$	$P = 0.97$	$P = 0.002$	$P = 0.06$						
Δ IL-12 72 / 0 HR	$r^2 = 0.21$	$r^2 = 0.04$	$r^2 = 0.004$	$r^2 = 0.007$	$r^2 = 0.02$	$r^2 = 0.12$	$r^2 = 0.28$	$P = 0.17$	$P = 0.41$	$P = 0.72$	$P = 0.81$	$P = 0.81$	$P = 0.61$
	$P = 0.01$	$P = 0.27$	$P = 0.75$	$P = 0.70$	$P = 0.48$	$P = 0.07$	$P = 0.43$						
Δ TGF-β 72 / 0 HR	$r^2 = 0.16$	$r^2 = 0.006$	$r^2 = 0.008$	$r^2 = 0.01$	$r^2 = 0.006$	$r^2 = 0.04$	$r^2 = 0.27$	$P = 0.17$	$P = 0.45$	$P = 0.36$	$P = 0.22$	$P = 0.85$	$P = 0.39$
	$P = 0.03$	$P = 0.69$	$P = 0.65$	$P = 0.58$	$P = 0.90$	$P = 0.27$	$P = 0.45$						

Multivariate regression analysis of gene expression using median age of blood transfused, transfusion requirement, selected markers of tissue injury, tissue ischaemia and age. Each univariate *P*-value and r^2 value is displayed. **Δ**, Indicates dynamic variables, as change in cytokine expression over time period displayed. *BD*, base deficit (at baseline (admission) and 24HR (24HR after injury). *ISS,* Injury Severity Score. *Quantity of PRBCs transfused*, number of units of packed red blood cells (PRBCs) transfused in first 24HR post-injury.

Supplementary Table 10 - Changes in mRNA levels from baseline to 24 hours following major abdominal surgery

Gene	Median baseline mRNA level	Median time 24 mRNA level	Fold change
IL-10	0.0000859	0.00034	3.96 (+)
FOXP3	0.00308	0.00136	2.26 (-)
GATA-3	0.0105	0.00375	2.8 (-)
IFN-γ	0.00021	0.0000866	2.42 (-)
IL-12	0.00036	0.00022	1.64 (-)
T-bet	0.0157	0.00661	2.38 (-)
TNF-α	0.00737	0.00495	1.49 (-)
IL-23	0.00093	0.00039	2.38 (-)
IL-27	0.01	0.0089	1.12 (-)
RORγT	0.00065	0.00025	2.6 (-)
TNF-α / IL-10	82.68	14.32	7.3 (-)

A comparison of the median mRNA levels at baseline and at 24 hours. mRNA levels are a relative quantification ratio between the candidate and the reference genes. (+), increasing mRNA levels; (-), decreasing mRNA levels. P value <0.01 for each comparison.

Supplementary Table 11 - Multivariate linear regression analysis for prediction of gene expression following major abdominal surgery

Response Variable		Predictor Variables Included in the model							Whole Model R^2	Predictors Independently Associated With Response Variable						
		Transfusion within 24 hours	Cancer diagnosis	Duration of surgery	Age	ASA class	Sex	Preop immune-suppression		Transfusion within 24 hours	Cancer diagnosis	Duration of surgery	Age	ASA class	Sex	Preop immune-suppression
FOXP3	***24HR***	***x***			***x***	***x***			**0.16**	**0.03**			**0.03**	**0.37**		
FOXP3	***48HR***	***x***	***x***	***x***	***x***				**0.16**	**0.05[1]**	**0.45**	**0.15**	**0.15**			
GATA-3	***24HR***	***x***			***x***		***x***	***x***	**0.13**	**0.23**			**0.03**		**0.23**	**0.03**
IL-12	***24HR***	***x***			***x***				**0.10**	**0.04**			**0.04**			
TNF-α	***48HR***	**x**			***x***				**0.06**	**0.39**			**0.04**			
IL-23	***24HR***	***x***		***x***	***x***	***x***			**0.23**	**0.25**		**0.0008**	**0.002**	**0.46**		
IL-23	***48 HR***	***x***	***x***	***x***	***x***				**0.17**	**0.37**	**0.28**	**0.01**	**0.05**			
RORγT	***24 HR***	***x***			***x***				**0.15**	**0.04**			**0.003**			
RORγT	***48 HR***	***x***	***x***		***x***				**0.19**	**0.05[1]**	**0.34**		**0.002**			
TNF-α / IL-10	***24HR***	***x***		***x***	***x***				**0.15**	**0.03**		**0.005**	**0.2**			
TNF-α / IL-10	***48HR***	***x***	***x***	***x***	***x***				**0.13**	**0.04[1]**	**0.39**	**0.03**	**0.27**			

Multivariate linear regression analysis of gene expression using requirement for blood transfusion, diagnosis of cancer, duration of the surgical procedure, age, ASA class and the presence of preoperative immunosuppression transfusion as potential independent variables. Each **x** represents an individual variable included in the model (when the univariate p value <0.1). Selection of variables is described in the methods section. The numbers under '*Predictors Independently Associated With Response Variable'* represent the *p*-values obtained when the model was run and are indicative of an independent association with the response variable. [1]Indicates *p*-value obtained following backwards elimination of non-significant variables.

Supplementary Table 12 - Multivariate Logistical Regression Analysis for Postoperative Infections following major abdominal surgery

Response Variable	Predictor Variables Included in the model			Whole Model R^2 (U)	Predictors Independently Associated with Response Variable		
	Cancer diagnosis	Duration of surgery	Transfusion within 24 hours postoperatively		Cancer diagnosis	Duration of surgery	Transfusion within 24 hours postoperatively
Postoperative infectious complications	***x***	***x***	***x***	**0.10**	**0.006**	**0.08**	**0.007**

Multivariate logistical regression analysis of postoperative infections using cancer diagnosis, duration of surgery and transfusion with the first 24 hours postoperatively as independent variables. Each **x** represents an individual variable included in the model (when the univariate p value <0.1) (Table 2). Selection of variables is described in the methods section. The numbers under '*Predictors Independently Associated With Response Variable'* represent the *p*-values obtained when the model was run and are indicative of an independent association with the response variable.

Supplementary Table 13 - Anti IL-6 cell culture patient sample demographics

Male	8 (100%)
Age (years)	64 (58 – 74)
ASA grade 3 or 4	1 (12.5%)
Operation details	
Elective	8 (100%)
Planned postoperative ICU admission	8 (100%)
Operation length (minutes)	296 (255 – 326)
Colorectal	2 (25%)
Upper GI	2 (25%)
HPB	4 (50%)
Laparoscopic	2 (25%)
Cancer	5 (62.5%)
Preoperatively immunosuppressed	2 (25%)
Nosocomial Infection	3 (37.5%)
Wound	2 (25%)
UTI	1 (12.5%)
Days post op infection (days)	11 (2-14)
Plasma IL-6 (ELISA) (pg/ml)	
Pre-Op	2.47 (2.25 – 3.08)
24hrs	64.91 (41.17 – 93.36)
48hrs	79.96 (30.33 – 86.11)

Data are described as medians with interquartile range or numbers with percentages in parenthesis. ICU, Intensive Care Unit. GI, Gasto-Intestinal. HPB, Hepato-Pancreato-Biliary.

Supplementary Table 14 - Anti IL-10 cell culture patient sample demographics

Male	11 (92%)
Age (years)	61 (58 – 72)
ASA grade 3 or 4	1 (12.5%)
Operation details	
Elective	12 (100)
Planned postoperative ICU admission	12 (100)
Operation length (minutes)	307 (240 – 319)
Colorectal	4 (33%)
Upper GI	4 (33%)
HPB	4 (33%)
Laparoscopic	2 (17%)
Cancer	9 (75%)
Preoperatively immunosuppressed	5 (42%)
Nosocomial Infection	4 (33%)
Wound	3 (25%)
UTI	1 (8%)
Days post op infection (days)	13 (4-16)
Plasma IL-10 (ELISA) (pg/ml)	
Pre-Op	1.0 (0.5 – 1.3)
24hrs	3.7 (2.5 – 5.2)
48hrs	2.2 (1.6 – 3.0)

Data are described as medians with interquartile range or numbers with percentages in parenthesis. ICU, Intensive Care Unit. GI, Gasto-Intestinal. HPB, Hepato-Pancreato-Biliary.

Supplementary Table 15 - Recombinant IL-10 cell culture patient demographics

Male	6 (75%)
Age (years)	58 (52 – 69)
ASA grade 3 or 4	2 (25%)
Operation details	
Elective	8 (100%)
Planned postoperative ICU admission	7 (87.5%)
Operation length (minutes)	275 (255 – 302)
Colorectal	2 (25%)
Upper GI	2 (25%)
HPB	4 (50%)
Laparoscopic	2 (25%)
Cancer	5 (62.5%)
Preoperatively immunosuppressed	1 (12.5%)
Nosocomial Infection	3 (37.5%)
Wound	1 (12.5%)
UTI	3 (37.5%)
Days post op infection (days)	22 (18-22)
Plasma IL-10 (ELISA) (pg/ml)	
Pre-Op	0.73 (0.61 – 1.1)
24hrs	11.0 (4.0 – 17.3)
48hrs	2.6 (2.1 – 4.1)

Data are described as medians with interquartile range or numbers with percentages in parenthesis. ICU, Intensive Care Unit. GI, Gasto-Intestinal. HPB, Hepato-Pancreato-Biliary.

Supplementary Table 16 - IFN-γ cell culture patient sample demographics

Male	5 (62.25%)
Age (years)	60 (52 – 69)
ASA grade 3 or 4	1 (12.5%)
Operation details	
Elective	8 (100%)
Planned postoperative ICU admission	8 (100%)
Operation length (minutes)	286 (225 – 362)
Colorectal	3 (37.5%)
Upper GI	2 (25%)
HPB	3 (37.5%)
Laparoscopic	3 (37.5%)
Cancer	6 (75%)
Preoperatively immunosuppressed	1 (12.5%)
Nosocomial Infection	2 (25%)
Wound	1 (12.5%)
UTI	2 (25%)
Pneumonia	1 (12.5%)
Intra abdominal	1 (12.5%)
Days post op infection (days)	15.5 (8 – 24)
Plasma IL-6 (ELISA) (pg/ml)	
Pre-Op	2.74 (2.08 – 3.33)
24hrs	47.31(27.02 – 176.90)
48hrs	25.88 (16.68 – 58.63)

Data are described as medians with interquartile range or numbers with percentages in parenthesis. ICU, Intensive Care Unit. GI, Gasto-Intestinal. HPB, Hepato-Pancreato-Biliary.

Supplementary Table 17 - GM-CSF cell culture patient sample demographics

Male	1 (12.5%)
Age (years)	62 (57.5 – 72.5)
ASA grade 3 or 4	2 (25%)
Operation details	
Elective	8 (100%)
Planned postoperative ICU admission	5 (62.5%)
Operation length (minutes)	163 (93 – 369)
Colorectal	3 (37.5%)
Upper GI	2 (25%)
HPB	3 (37.5%)
Laparoscopic	2 (25%)
Cancer	6 (75%)
Preoperatively immunosuppressed	1 (12.5%)
Nosocomial Infection	2 (25%)
Wound	0 (0%)
UTI	1 (12.5%)
Days post op infection (days)	10.5 (10-11)
Plasma IL-6 (ELISA) (pg/ml)	
Pre-Op	3.6 (2.7 – 5.1)
24hrs	47.2 (18.0 – 150.1)
48hrs	41.5 (22.0 – 161.3)
Plasma IL-10 (ELISA) (pg/ml)	
Pre-Op	0.9 (0.8 – 1.1)
24hrs	3.6 (2.2 – 4.3)
48hrs	2.6 (1.9 – 9.0)

Data are described as medians with interquartile range or numbers with percentages in parenthesis. ICU, Intensive Care Unit. GI, Gasto-Intestinal. HPB, Hepato-Pancreato-Biliary.

Supplementary Protocol 1: HEMS pre-hospital transfusion protocol.

Pre-hospital Care Standard Operating Procedure

Pre-hospital blood transfusion

REVIEW:	January 2014	
APPROVAL/ ADOPTED:	LAA Policy Board	
DISTRIBUTION:	LAA Doctors LAA Paramedics Barts Health Trust Transfusion committee London Trauma Haematology Group	
RELATED DOCUMENTS:	Code Red SOP Haemorrhage control, vascular access and fluids SOP Packaging SOP Equipment resource folder – Golden Hour box™, Belmont buddy lite™ fluid warming system Barts Health Code RED flow chart	
THIS DOCUMENT REFERS TO:	☑ PHC Clinical Practice PHC Non-clinical Practice PHC Operational Procedure	Ref: CP-8
REVIEW:	January 2014	
APPROVAL/ ADOPTED:	LAA Policy Board	
DISTRIBUTION:	LAA Doctors LAA Paramedics Barts Health Trust Transfusion committee London Trauma Haematology Group	
RELATED DOCUMENTS:	Code Red SOP Haemorrhage control, vascular access and fluids SOP Packaging SOP Equipment resource folder – Golden Hour box™, Belmont buddy lite™ fluid warming system Barts Health Code RED flow chart	
THIS DOCUMENT REFERS TO:	PHC Clinical Practice PHC Non-clinical Practice PHC Operational Procedure	Ref: CP-8

Aim:

To provide pre-hospital blood transfusion to trauma patients whilst complying with local and national legislation and avoiding wastage of blood products.

Objectives:

- Describe the arrangements for the availability of emergency blood for pre-hospital teams.
- Describe the indications for pre-hospital blood transfusion.
- Describe the process for pre-hospital blood transfusion.

Background:

Approximately 10% of London HEMS patients will trigger the Code RED protocol. These patients require early administration of blood products and rapid vascular control if they are to survive. In addition, patients in traumatic cardiac arrest may benefit from blood transfusion if hypovolaemia is considered to be a contributing factor.

Principles:

- Emergency blood is a precious and limited resource.
- Blood transfusion is not a replacement for careful packaging and splintage.
- LAA teams will ensure all efforts to preserve blood volume are maximised prior to and during emergency blood transfusion.
- LAA carries blood in accordance with NHSBT policies and guidance.
- The duty PHC Consultant on-call should be contacted prior to pre-hospital transfusion in all cases.
- All units of blood must be fully traceable via documentation and database records.
- All Code RED patients should be given warmed blood.
- Tranexamic Acid 1g bolus should be given as per Code RED protocol.
- LAA teams will avoid wastage of blood.

Policy:

Indications to transfuse pre-hospital emergency blood

- Traumatic cardiac arrest where hypovolaemia is judged to be a contributing factor.
- Code RED patients (see SOP Code RED) where volume resuscitation is deemed necessary prior to arrival at hospital.

Storage of pre-hospital emergency blood:

- Emergency blood is packed into the Golden Hour box™ and sealed with a tag by Barts Health transfusion staff.
- Each box contains 4 units of O negative emergency blood and a data logger.
- The outer carry bag contains transfusion paperwork and a pre-labelled blood bag from the pre-transfusion sample.
- The outer carry bag should be sealed by the transfusion staff with a tag to indicate that the contents are complete and unused.
- Golden hour boxes™ can maintain a steady state temperature of 2-4C for 48-72 hrs.
- Golden hour boxes™ should be returned to the transfusion lab every 24 hours for repacking.
- All boxes must remain sealed until the LAA doctor declares "Code RED and intention to transfuse".

Location of Golden Hour boxes:

- Pre-packed Golden hour boxes™ are available to all operational teams. Boxes are kept in the aircraft and in the boot of operational trauma response vehicles.
- Spare pre-packed Golden Hour boxes™ are available from the transfusion department.

In-flight decision-making:

- The medical team should make a decision based on information available whether to take the blood to scene as a primary action. They should consider the information from EOC, the mission run sheet and the overhead view of the incident.

On scene:

- If blood transfusion is deemed necessary and the blood box is in the aircraft, the team should either ask the pilots to bring the blood box to scene or a member of the emergency services may be dispatched to retrieve the blood box from the aircraft.

- Obtain pre-transfusion blood sample in a Group & Screen (cross match) bottle (BD purple long tube) and a clotting sample (blue bottle) for ROTEM analysis at RLH. Paediatric bottles (red top) are available for infants. These bottles are acceptable to all London MTC transfusion laboratories.

- The pre-transfusion blood sample should be drawn from an unflushed cannula or a femoral stab sample if IV access has already been obtained and flushed with saline.

- The doctor must sign, date and time the sample immediately. This blood sample should be kept in a safe place (doctor's pocket) to be handed over at the MTC.

The Golden Hour box™ must only be opened when the decision has been made to transfuse blood.

- Break the seal and open the Golden Hour™ box.
- Keep the enclosed paperwork safe.

- Remove required no of units and close box immediately. This will avoid wastage of unused units. Unused units can be returned to the lab and reallocated for use within Barts Health NHS trust.

- Check units of blood using challenge and response: perform visual inspection of unit (look for discolouration, haemolysis, large clots), confirm blood type (O neg) and expiry date. At least one member of the LAA team must perform the visual blood inspection

- Transfuse emergency blood via Belmont buddy lite™ warming system at an appropriate rate based on patient physiology and to allow blood to be heated.

- Contact MTC and place pre-alert call stating HEMS team call sign, CAD no, gender, and mechanism of injury. State that patient meets Code RED criteria and pre-hospital blood transfusion is ongoing.

Patient labelling:

- A "pre-hospital blood transfusion" patient wristband must be placed on the patient at the time of transfusion.

- Each wristband has a unique number, which can be recorded on the transfusion record sheet along with the LAS CAD number and patient details.

Blood unit labelling:

- Each unit of blood has two labels to identify the unit

    - Paper tear off tag
    - Main label on front of unit

- The "tear off tag" must be completed, signed and placed in the Golden Hour box™ when the unit is transfused and returned to the Royal London Hospital transfusion laboratory with the original transfusion record sheet.

- The main label remains on the unit of blood and the empty blood bag can be disposed of in a yellow incineration bin by hospital staff when transfusion has finished.

Transfusion record sheet:

- The transfusion record sheet is an A4 form, which is found inside a plastic pouch inside the blood box carry bag.
- The completion of this form is mandatory to ensure 100% traceability for every unit of blood.
- Transfusion staff will complete part of the form with the unique ID for each unit of blood when the box is packed.
- LAA staff must complete the remaining fields after transfusion has occurred.
- **Please check carefully that the unit number on the label and stickers matches the unit number on the blood bag and also correlates with the paperwork – there is potential for human error at every step of the labelling process.**
- This form must be photocopied at the MTC and handed over directly to the Trauma Team Leader along with the photocopied mission record sheet and observations.
- The original transfusion record form must be returned in the Golden Hour box to Barts Health transfusion lab with the tear off labels.
- The date, time, CAD number and patient destination (e.g. St Mary's MTC, King's MTC, mortuary) must be recorded on this form. This form must be signed by the LAA doctor.

Handover at MTC:

- The verbal handover must explicitly state that pre-hospital transfusion has occurred.
- Ensure the patient wristband is still attached to the patient.

- The doctor must complete and sign the cross match form at the MTC and transcribe the MTC patient details on to the blood bottle.

- The cross match request form sent to the MTC laboratory should be annotated to indicate that this sample has been taken prior to pre-hospital transfusion.

- The LAA transfusion record sheet, cross match bottle and request form must be handed to the Trauma Team Leader in the pre-labelled bag. It is their responsibility to check the details and take responsibility to send the sample to the lab.

- The MTC should repeat the Group & Screen on arrival as per MTC protocol.

- Unused units of blood must not be left with the MTC but must be returned in the blood box to RLH.

HEMS Database:

- The HEMS database must be completed using the drop down intervention menu. Include as many of the following as are relevant:

    - Code Red
    - Blood (state no of units given)
    - Tranexamic Acid 1g
    - Buddy lite fluid warmer

- The free text section should include the unique unit numbers of the units transfused.

- Documentation must be complete in order to provide 100% traceability for all units of blood.

Incident reporting:

- Any incidents arising from Pre-hospital emergency blood transfusion must be reported via the Barts Health Datix system in addition to the local helipad reporting system.

How to arrange resupply of pre-hospital emergency blood:

When a Golden Hour box™ has been opened it must be returned to Barts Health transfusion laboratory as soon as possible. The transfusion record sheet and tear off tags must be returned in the same box.

- The transfusion lab should be informed when the mission is complete via ext 61117 or bleep 1422 out of hours.

- State which number box has been opened and request a replacement.

- A spare Golden Hour box™ may be available on the helipad.

- RLH porters will return and collect Golden Hour boxes™. Porters can be contacted on ext 40839 or bleep no 1213 / 1316 / 1613. You should state the number and location of the used box, which needs to be returned to the lab. You should also state where the replacement box should be taken to ie helipad.

References:

1. The Blood Safety and Quality Regulations 2005 no .50 (MHRA)

http://www.legislation/gov/uk/uksi/2005/50/contents/made

2. Barts Health NHS Trust. Blood Transfusion Policy. BLT/POL/11109/N&Q

http://bltintranet/Policiesandguidelines/Blood%20Transfusion%20Policy.pdf

3. Barts Health NHS Trust Code Red Adult flow chart.

Appendix:

1. Validation graph for Golden Hour box
2. LAA Transfusion record sheet
3. Blood box changeover record sheet
4. Label for pre-transfusion sample bag

Supplementary Protocol 2: Royal London Hospital Code Red protocol.

CODE RED TRAUMA – guidance notes July 2011

Only a senior member of the Trauma Team who has undergone induction training can trigger CODE RED TRAUMA

Tranexamic acid:

Tranexamic acid is an antifibrinolytic agent that inhibits activation of plasminogen to plasmin; the latter is responsible for fibrin degradation

In trauma-induced massive haemorrhage a bolus of Tranexamic acid (1g, iv over 10 min) should be started within 3 hours of injury followed by continuous infusion (1g, iv) over 8 hours

Blood Transfusion – Emergency Group O Stock:

Group O Neg (use in females) and O Pos (use in males) blood available in blood track fridge for CODE RED use

MUST INFORM Blood Bank when units used so that stocks can be replaced

Switch from Group O to patient's group as soon as possible to minimize use of Gp O

CODE RED PACK A contains:

6 units Red Cells

4 units FFP.

CODE RED PACK B contains:

6 units RBC

4units FFP

1 unit Platelets

2 pool cryoprecipitate.

Fresh Frozen Plasma:

FFP needs defrosting before issue – this takes around 30mins (allow time for transit)

Each dose should contain ~15ml/kg of FFP – around 4units for average adult

Cryoprecipitate:

Cryoprecipitate needs defrosting before issue – this takes around 30mins (plus allow for time transit) Do not put cryo in fridge after defrosting since can precipitate

Each dose should contain 2 pools cryoprecipitate for average adult

Platelets:

Stored at ambient temperature DO NOT REFRIGERATE

Since platelets have short shelf life of 5 days only the blood bank has limited supplies of platelets and has to order additional units from the Blood centre as needed.

Laboratory testing:

Must request fibrinogen as well as coagulation screen and Full Blood count for all patients with massive haemorrhage

Repeat testing of Coagulation screen including fibrinogen and platelet count needed after transfusion of components to guide further replacement.

Near Patient Testing

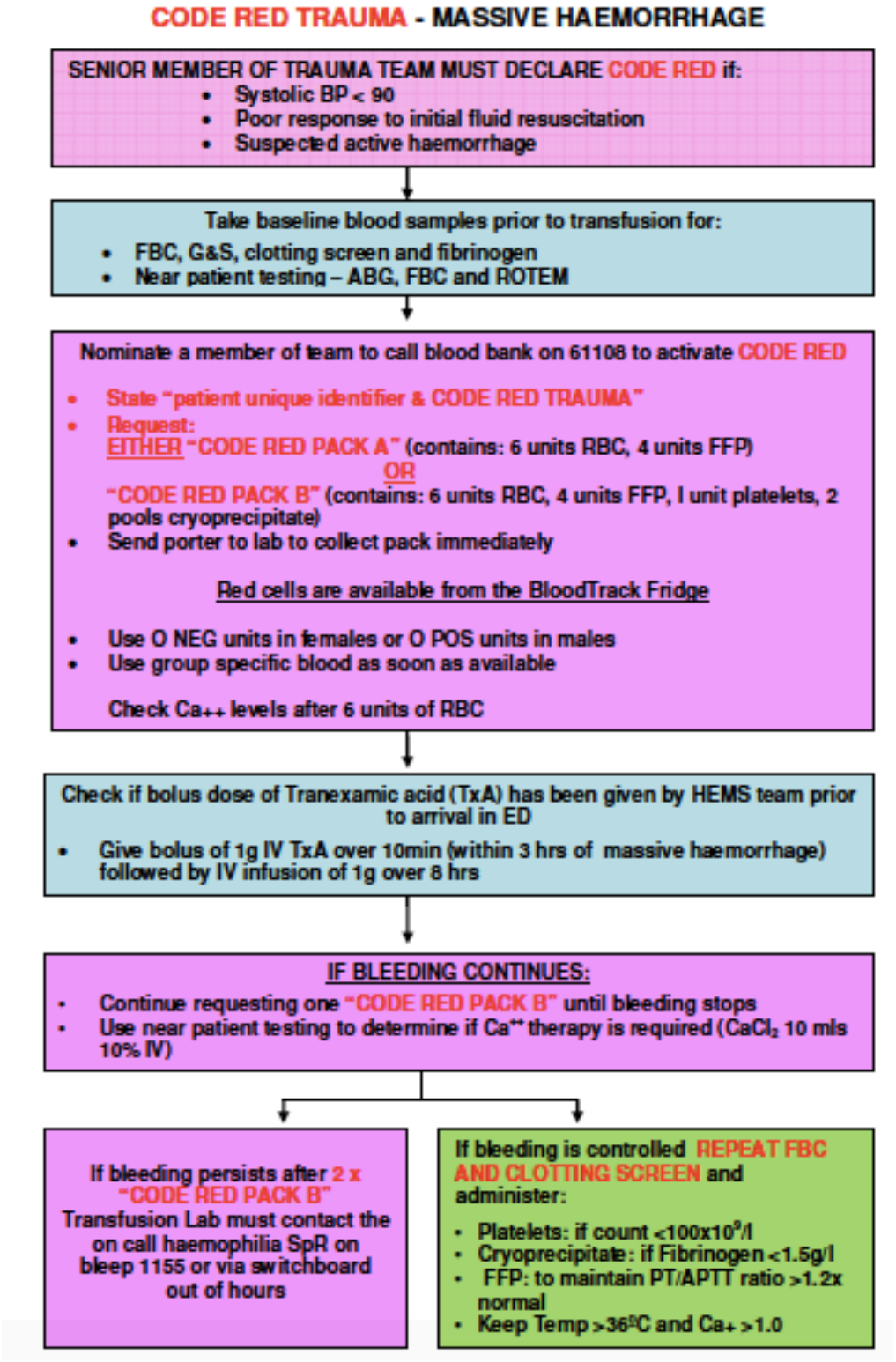
Any near patient testing device used MUST comply with the Trust Point of Care Testing policy.

Recombinant FVIIa:

Discuss with Haemophilia Registrar (Bleep 1155 or via switchboard on call) regarding use of rFVIIa in patient who continues to bleed despite replacement therapy with FFP, platelets and cryoprecipitate.

TRACEABILITY IS ESSENTIAL FOR ALL UNITS TRANSFUSED

Supplementary Protocol 3: Royal London Massive Transfusion Protocol.



Supplementary Protocol 4: HEMS Code Red guidance.

Trauma Service Standard Operating Procedure

Code Red Trauma Team response

(Tier 3 trauma response)

REVIEW:	January 2017	
APPROVAL/ ADOPTED:	RLH MTC Policy Board	
DISTRIBUTION:	All Trauma Team members (Tier 1, 2 and 3)	
RELATED DOCUMENTS:	Roles and responsibilities of Trauma Team Leaders SOP - Trauma Team Response SOP - REBOA Code Red policy	
THIS DOCUMENT REFERS TO:	☑ Clinical Practice Non-clinical Practice Operational Procedure	

Aim:

To provide an explanation of how the assessment, care and initial treatment of Code Red trauma patients should be delivered in order to optimise outcome.

NB. All team members must be familiar with the Trauma Team Response SOP prior to reading this document.

Objectives:

- To achieve consistent Code Red trauma team performance.
- To describe the expected preparation for Code Red trauma patients after pre-alert.
- To describe the expected reception of Code Red trauma patients.

Overview:

”Code Red” is the term used to alert the Major Trauma Centre that a trauma patient is at risk of death from serious haemorrhage. In many cases this is due to non-compressible haemorrhage and damage control surgery or interventional radiology techniques will be required to achieve vascular control.

The Royal London Emergency Department activates the Code Red protocol around 150 times per year. Activation will mobilise Consultant level attendance for Trauma Surgery and Anaesthesia. Specific departments (transfusion, theatre, Interventional Radiology) will take action to ensure the incoming patient is given the best chance of survival.

Principles:

- Code Red patients have a high mortality (up to 50%).
- Code Red patients require additional expertise and resources to be immediately available.
- Code Red patients are likely to require rapid transfer to another department eg theatre / IR within minutes of arrival. Transfer equipment must be prepared in advance of the patient’s arrival with this in mind.
- Code Red trauma calls must be led by an ED Consultant or by a suitably trained registrar / fellow under direct supervision of the ED consultant. Inexperienced team leaders must not be left unsupervised as Team Leader for Code red patients.
- Code Red trauma calls will attract additional staff and interest. Crowd control is the responsibility of the TTL and is essential to optimise team performance and patient outcome.
- An in-bound pre-hospital team may declare Code Red or the TTL may decide to declare Code Red based on information received from the Ambulance Service or transferring

hospital team. Code Red may also be activated if a patient deteriorates after arrival in order to provide an escalated response from the "Advanced" trauma call.

- All REBOA pre-alerts require a Tier 3 Code Red trauma team response.

Policy:

1.0 Code Red Pre-alert and Blue call

- A pre-hospital doctor (usually from an Air Ambulance team) will pass a pre-alert message via the dedicated phone in resus to warn of a Code Red patient.
- The initial pre-alert will only contain brief details ie age, gender, mechanism, code red request. This limited information allows additional preparation time prior to the more detailed blue call.
- Details should be recorded on the paper forms provided next to the phone.
- The senior nurse in resus and the allocated senior doctor must be informed immediately.
- The Code Red Trauma Team should be activated via switchboard (ext 2222) 15 minutes before the estimated time of arrival (ETA).
- Switchboard will state "Code Red trauma team to A&E resus" over the bleep system.
- All team members should make their way immediately to the resus room.

2.0 Trauma Team arrival and etiquette

On receipt of the initial pre-alert or the full blue call (whichever is earliest) the following actions should be carried out:

- Request trauma pack from reception – a unique identifier is required to order blood products, blood tests and imaging.
- The TTL role is to provide oversight of the preparation phase and to ensure that all team members have a clear understanding of their role. The TTL must pay attention to detail and try to anticipate events.
- The TTL must use the Code Red checklist to ensure all actions are completed in good time.
- All attending members of the Trauma Team must "sign-in" the trauma booklet and don lead aprons, plastic over-aprons and gloves. Eye protection is available.
- Every team member must read their allocated action card and check they have necessary equipment to perform their role.

3.0 Trauma Bay preparation for Code Red trauma call

- Nurse 1, Nurse 2 and the Operating Department Practitioner play a vital role at this stage and as a team must ensure that the designated bay is fully prepared for the reception of the patient.
- Preparation must include anticipation of immediate needs, urgent procedures and rapid transfer to other departments eg theatre / IR .
- Pre-arrival information typed on word document on computer screen in large font.
- Trauma booklet with sticker on every page and front sheet attached securely. Scribe allocated.
- Resus trolley in central position in bay, positioned at appropriate height with sides in "down" position and sheet laid out.
- Theatre lights x 2 switched on and positioned over trolley.
- Patient gown, sheet and blanket available.
- 2 name bands with unique identifier details.
- Tympanic thermometer and Bair Hugger (machine and sheet) available.
- Monitoring ready – ECG dots attached to leads, variety of BP cuffs, oxygen saturation probe, oesophageal temperature probe, EtCO2 etc.
- Belmont fluid warmer primed and loaded with blood products (TTL discretion as to PRBC or plasma initially, depending on pre-hospital treatment). IV long extension set available.
- Ultrasound machine in position switched on and gel available. Operator allocated (radiologist).
- Anaesthetic equipment – anaesthetic machine checked, 2 oxygen sources, airway kit laid out (including airway adjuncts and BVM) if not already intubated, suction working, emergency drug tray, propofol infusion and pump, RSI checklist available.
- Trolley with IV access and venepuncture equipment, blood bottles including research samples. If the patient is ventilated, an femoral arterial stab should be performed to obtain an arterial blood sample.
- Sterile procedures preparation – an appropriately trained and competent doctor should be scrubbed up and in a surgical gown if it is anticipated that an invasive or surgical procedure eg. central line, thoracostomy, thoracotomy or REBOA may be required urgently.
- Transfer kit available – portable monitor, ventilator, full oxygen cylinder, suction, transfer bag, bridge for trolley, P1 card.

In addition, for patients arriving by helicopter –

- 2 units PRBC (from resus fridge) and name bands sent to helipad with porter.
- Resus room and corridor doors should be held open by spare personnel to assist teams in progressing rapidly from lift core 4 to the resus room.
- Trolley should be removed from bay for patients arriving by air. The helicopter team will deliver the patient on a resus trolley directly from the helipad.

4.0 Pre-arrival briefing

Prior to arrival of the patient, the TTL should brief the team. This is essential to check that everyone has the required equipment and is clear about their role. Briefing should be concise but should cover the following points:

- Introduction of all team members (first names) and roles.
- Confirmation of pre-alert info eg mechanism of injury, expected injuries and condition.
- Confirmation of mode of transport eg by land or air.
- Ensure all team members are dressed in PPE / sterile gown and gloves.
- Ensure all team members have required equipment.
- Ensure any specific actions or roles are allocated eg chest drain insertion.
- Confirm completion of all tasks on the Code Red checklist.

5.0 Handover

- The Pre-hospital team will deliver a verbal handover to the trauma team. This usually takes place after the patient has been transferred onto the resus trolley but in certain circumstances may be preceded by a request for immediate action eg. start blood transfusion or to perform a procedure eg. thoracotomy.
- All pre-hospital documentation (patient report form, mission record and observations) must be secured in the front of the trauma booklet.

6.0 Code Red drugs

Code Red patients may require the delivery of rapid anaesthesia in order to deliver invasive intervention.

- The ODP / anaesthetist will collect the emergency anaesthetic drug tray from the resus drug fridge (mounted on wall).
- Prepare anaesthetic drugs (usually ketamine and rocuronium), sedation (propofol) and analgesia (morphine / fentanyl).
- Ensure the following additional drugs are available in the trauma bay:

1. Tranexamic acid 1g bolus and 1g infusion dose (to prevent hyperfibrinolysis)
2. Calcium chloride 10ml 10% (to treat hypocalcaemia and hyperkalaemia, also acts a positive inotrope)
3. Insulin (Actrapid vial) and 50ml 50% dextrose (to treat hyperkalaemia)

7.0 Acute Traumatic Coagulopathy (ATC)

Code red patients are at risk of Acute Traumatic Coagulopathy. This coagulopathy is triggered by tissue damage and hypoperfusion (shock). High injury load and / or profound hypotension should lead to suspicion that ATC may be present. Blood gas analysis is useful to predict the need for massive transfusion eg BE > - 6 and raised lactate suggest that ATC is likely.

ROTEM analysis can confirm or exclude the diagnosis of ATC in under 5 minutes. ROTEM analysis is available at RLH. The analyser is kept in theatres but is mobile and can be moved to resus or IR if required. Trained operators include ODPs, Trauma Service research team and selected anaesthetists / intensivists.

ATC will be exacerbated by other causes of coagulopathy eg hypothermia, acidosis and dilution of clotting factors. Steps must be taken to prevent worsening of the patient's condition:

- Do not give crystalloid or colloid unless explicitly instructed to do so by TTL.
- Measure core temperature in ventilated patients using an oesophageal temperature probe.
- Maintain normothermia – keep the patient covered up, use Bair Hugger if temp <36C, ensure all blood products are given via fluid warmer eg Belmont.
- Check ionised calcium levels (ABG) and correct as required. Calcium levels may be low in severely injured patients and those receiving blood transfusions.
- Confirm whether pre-hospital team has given Tranexamic Acid (TXA) 1g bolus. If not, 1g should be administered, provided it is still within 3 hours of injury.
- Commence TXA infusion 1g in 100ml 0.9% saline over 8 hours.

- If ROTEM is available use result to guide blood product therapy.

8.0 Radiology services

8.1 Radiologist

- A radiologist should attend Code Red trauma calls. They can be contacted via DECT 45709 or bleep xxxx.
- This role will allow face-to-face communication regarding initial diagnostic findings and ensure accurate protocols for further imaging.
- The radiologist should be present in the CT suite at the time of scanning to assist in rapid decision-making and to provide a verbal "ABCD – primary survey report".
- A more detailed, structured electronic report will be provided within 60 minutes. Any updates to reports should be communicated to the Duty ED Consultant on DECT 45722 or via bleep 1115.

8.2 Interventional Radiology

- Interventional Radiology (IR) is provided at the Royal London by a group of 6 Consultants and a pool of nursing staff.
- IR has an increasing role in the management of Code Red patients and should be available within 30 minutes at a Major Trauma Centre.
- The Duty IR Consultant should be informed immediately if pre-alert information suggests that IR may be required eg serious pelvic injury

  - Mon-Fri 9-5 ext 40105

  - Out of hours and weekends via switchboard.
- All REBOA activations require IR presence in resus. This is to ensure rapid senior decision making between TTL, Trauma Surgery and IR Consultants. TTL should inform Duty IR Consultant as soon as REBOA pre-alert is received.
- Anticipation and early activation will allow the IR suite to be prepared, on-call staff to proceed to RLH and ensure patients receive timely intervention.

- TTL will decide with assistance from colleagues whether transfer to IR is required. The whole team should accompany the patient to the IR suite and should anticipate and be prepared for deterioration in the patient's condition.

8.3 Imaging requests on CRS

- Detailed imaging may not be prudent in Code Red patients and critical decision-making is often made based on physiology and suspected injuries.

- Initial imaging requests should be placed on CRS as soon as the trauma pack is received. As much detail as possible should be provided in the clinical details section. Requests will be based on mechanism.

- A polytrauma patient may require:

  - CXR

  - PXR

  - CT head

  - CT cervical spine

  - CT chest, abdomen, pelvis

8.4 Imaging during Code Red trauma calls

- Plain film imaging is useful in unstable patients to exclude unexpected diagnoses eg haemothorax, open book pelvis. Remember the reported mechanism may not represent the whole story eg; stab to chest may also have fallen from height.

- E-FAST in the hands of an experienced operator can be useful to ascertain which cavity presents the greatest threat to life eg intra-abdominal FAST positive may expedite transfer to theatre, pericardial tamponade may expedite ED resuscitative thoracotomy. A negative result however, does not confirm the absence of pathology.

- E-FAST is helpful to identify pneumothorax in supine patients. This modality is more sensitive than plain CXR to detect this pathology.

- TTL will decide with assistance from colleagues whether CT is appropriate in an unstable patient. In the absence of an obvious indication to perform an immediate procedure, transfer to theatre or IR, it may be prudent to undertake CT imaging. The whole team should accompany the patient to the CT suite and should anticipate and be prepared for deterioration in the patient's condition.

9.0 Transfusion

- Nurse One is responsible for informing the Transfusion lab of a Code Red trauma call. A dedicated telephone in resus provides a direct line to the laboratory.

- TTL must indicate to Nurse One which blood products are required. The initial request is usually for Pack A only (PRBC and plasma). Pack B (PRBC, plasma, platelets and cryoprecipitate) can be requested at the same time if required.

- Packed Red Blood Cells are available in the resus blood fridge (12 units O Neg, 12 units O positive).

- Plasma must be collected from the lab – a minimum time of 20 minutes is required to thaw this product.

- A porter should be sent directly to the Transfusion lab (4^{th} floor pathology building) to wait for the thawed plasma to be made available. The porter will then bring the plasma to the resus room.

- All blood products must be prescribed and timed on the Blood Product prescription page in the trauma booklet.

- All blood products must be checked by 2 members of staff prior to infusion.

- Nurse One must inform the lab if the patient moves to theatre or IR.

- Any Code Red patient being transferred to theatre or IR should have Pack B requested prior to leaving ED.

- An allocated nurse must complete the Code Red blood product tally document in real-time.

- All blood products must be delivered through a warming system.

See appendix for Code Red guideline.

10.0 Blood test requests

10.1 Trauma panel and near patient testing

- "Trauma panel" blood requests should be placed on CRS as soon as the trauma pack is received.
- Request immediate cross-match 6 units and generate 2 paper forms (one for HEMS sample and one for ED sample).
- Near patient testing machines should be used for blood gas and FBC analysis.
- A blood gas sample should be analysed as soon as possible – check base deficit and lactate. Take an arterial sample in intubated patients to correlate EtCO2 and PaCO2.
- If the ROTEM machine is available, run EXTEM and FIBTEM channels.

10.2 Pre-transfusion samples

Once several units of blood have been transfused it may be impossible to determine the native blood group due to a mixed field sample. Every effort must be made to obtain a pre-transfusion sample.

- The HEMS team may provide a pre-transfusion sample. This must be clearly labelled as "HEMS sample" on the paper form. The HEMS doctor must complete patient details on the sample and sign the bottle and the paper form.
- The trauma team should provide a second sample as confirmation at the time of venepuncture. This should be labelled as the "ED sample" on the second paper form.
- Occasionally it is not possible to obtain a sample prior to transfusion. The lab should be informed and a sample should be sent as soon as it is available.

11.0 Emergency reversal of Warfarin and Novel Oral Anti-Coagulants (NOAC)

- Near-patient testing (Coaguchek) is available to check INR. This is useful if a patient is suspected / or confirmed to be on warfarin. A result will be available within 20 seconds.
- The Coaguchek machine is kept in the Near Patient testing room near resus.
- Prothrombin Complex Concentrate (PCC) - Octaplex is kept in the Code Red cupboard in resus for the emergency reversal of warfarinised patients.
- PCC may also be useful for patients on NOACs with severe bleeding. Discussion with the Duty Haematology Consultant is advised for this group of patients.

12.0 Transfer to CT / interventional radiology / theatre.

- Communication - prior to any transfer, the destination receiving team eg CT / theatre / Interventional radiology, must be made aware of the patient's imminent arrival.

- Priority 1 lift pass is kept in the Code Red cupboard (near bay 8) to facilitate rapid transfer to theatre / IR.

13.0 Documentation and verbal handover

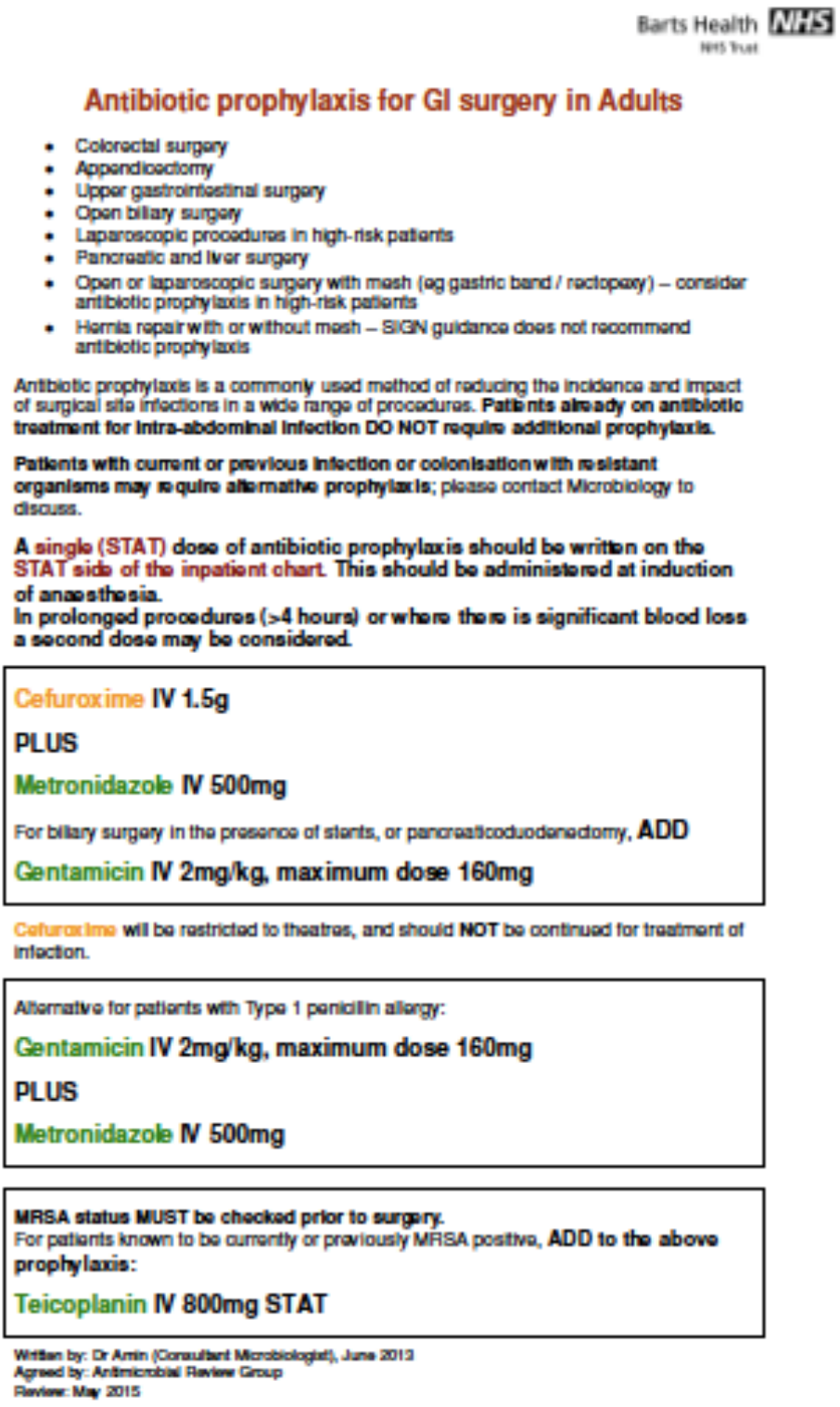
TTL must ensure that the medical notes are an accurate reflection of the events to date.

An explicit verbal handover to an appropriately senior colleague should take place at an appropriate time. This handover must be recorded in the notes.

14.0 Code Red debrief

In order to identify good practice and opportunities for improvement, it is useful to offer a debriefing process following Code Red trauma calls. This will usually occur several hours after the event due to key personnel being engaged in procedures. A recommended template and guidance is provided on the Q drive. Any issues arising can be forwarded to the relevant specialty lead, the MTC Trauma Director or the Trauma Governance Lead.

Supplementary Protocol 5: Royal London Hospital Prophylactic Antibiotics Guidelines



Barts Health NHS
NHS Trust

Antibiotic prophylaxis for GI surgery in Adults

- Colorectal surgery
- Appendicectomy
- Upper gastrointestinal surgery
- Open biliary surgery
- Laparoscopic procedures in high-risk patients
- Pancreatic and liver surgery
- Open or laparoscopic surgery with mesh (eg gastric band / rectopexy) – consider antibiotic prophylaxis in high-risk patients
- Hernia repair with or without mesh – SIGN guidance does not recommend antibiotic prophylaxis

Antibiotic prophylaxis is a commonly used method of reducing the incidence and impact of surgical site infections in a wide range of procedures. **Patients already on antibiotic treatment for intra-abdominal infection DO NOT require additional prophylaxis.**

Patients with current or previous infection or colonisation with resistant organisms may require alternative prophylaxis; please contact Microbiology to discuss.

A single (STAT) dose of antibiotic prophylaxis should be written on the STAT side of the inpatient chart. This should be administered at induction of anaesthesia.
In prolonged procedures (>4 hours) or where there is significant blood loss a second dose may be considered.

Cefuroxime **IV 1.5g**

PLUS

Metronidazole **IV 500mg**

For biliary surgery in the presence of stents, or pancreaticoduodenectomy, **ADD**

Gentamicin **IV 2mg/kg, maximum dose 160mg**

Cefuroxime will be restricted to theatres, and should **NOT** be continued for treatment of infection.

Alternative for patients with Type 1 penicillin allergy:

Gentamicin **IV 2mg/kg, maximum dose 160mg**

PLUS

Metronidazole **IV 500mg**

MRSA status MUST be checked prior to surgery.
For patients known to be currently or previously MRSA positive, **ADD to the above prophylaxis:**

Teicoplanin **IV 800mg STAT**

Written by: Dr Amin (Consultant Microbiologist), June 2013
Agreed by: Antimicrobial Review Group
Review: May 2015

If there is pus found in the abdomen, an acutely inflamed appendix, or bowel perforation has occurred, institute a therapeutic course of antibiotics **as per treatment guidelines** for intra-abdominal sepsis.

References:

1. National SIGN guidelines on antibiotic prophylaxis in surgery (2008). http://www.sign.ac.uk/pdf/sign104.pdf
2. *Antibacterial prophylaxis in surgery: I – Gastrointestinal and biliary surgery*. DTB 2003;41:89-96